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Doctor of Philosophy Degree
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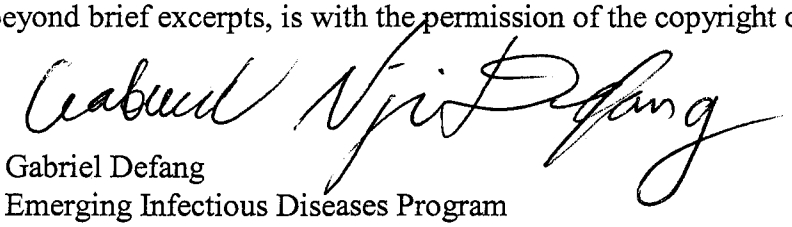

Gabriel Defang
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ABSTRACT

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Doctor of Philosophy

2007

Thesis directed by: CAPT Gerald V. Quinnan, Jr., M.D. Chair, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences

Title of Dissertation:

Toward Viral Vaccine Development: A modified Venezuelan Equine Encephalitis Replicon As A Strategy For Optimizing Immunogenicity.

Alphaviral vectors have been used as vaccines for immunization against several viral infections. However, these vectors typically induce rapid cytopathic effects in mammalian cells. The cytopathicity of these vectors allow for only transient expression of transgenes. In viral models where long-term expression may be needed to induce adequately differentiated immune responses, the current wild-type alphaviral vectors may not be sufficiently immunogenic. This thesis describes development of attenuated, prolonged-expression, Venezuelan equine encephalitis virus (VEE) vectors by modifying the non-structural protein-2 gene (NsP2) through the introduction of single or double point mutations.We studied the potential of wild-type and modified VEE replicon particles (VRPs) for optimizing and enhancing the immunogenicity of HIV-1 envelope glycoprotein and to the fusion (F) and attachment (G) glycoproteins of two zoonotic pathogens, Hendra and Nipah viruses, in a murine and rabbit model.

We demonstrate in this study that modified VRPs are efficient in optimizing neutralizing antibody responses. Three-fold lower doses and only two vaccinations of modified VRPs were sufficient to induce maximal HIV-1 neutralizing antibody responses in mice compared to three vaccinations of wild-type VRPs.We also report here for the first time that, high-level cross-reactive neutralizing antibodies against henipaviruses were achieved using VRPs. Overall, these data suggest that the use of modified alphavirus-based vaccine platforms should be given consideration for the development of viable antiviral vaccines.

Toward Viral Vaccine Development: A modified Venezuelan Equine Encephalitis Replicon as a Strategy for Optimizing Immunogenicity.

By

Gabriel Nji Defang

Thesis/dissertation submitted to the Faculty of the Department of Emerging Infectious Diseases Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2007

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My deepest gratitude to the entire EID faculty for the vital role they have played in my education.

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To the Quinnan lab, you are the best team. Thank you, Peng Fei, for your advice and support throughout my time as a graduate student. Ming, Zhiqiang, Ximan and Claire, thanks for the technical assistance. Fatim, thanks for being there for me in good times and in bad times. Peter, thanks for making life in the lab a lot easier for me.

DEDICATION

This dissertation is dedicated to my parents Fuatabong Defang and Mary Anjoh Defang who never stopped believing in me. They are my greatest inspiration.

To my brothers Hans, Ngunyi and Asongamin and my sisters Nkeng and Fualefeh for their unwavering support.

To my daughter –the jewel of our family; Princess Chelsea, for the joy she brings into my life.

To my beloved wife, Benita for her unconditional love.

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CHAPTER 1: GENERAL INTRODUCTION

LITERATURE REVIEW

I. Brief History of VEE

The New World alphavirus, Venezuelan equine encephalitis virus (VEE) was initially isolated on March 3, 1938 in a post-mortem brain specimen from a horse following an outbreak of equine encephalitis in the Guajira Peninsula of Northern Columbia and Venezuela that began in 1936 (11, 91). This virus has established itself as a unique member in the genus *Alphavirus* of the *Togaviridae* family. It is considered the most important human and equine pathogen in this family. Natural transmission of VEE among individuals is by a mosquito vector (166). However, transmission by aerosol exposure has occurred in the laboratory setting (150). Consequently, VEE has a recognized potential for weaponization, and has been categorized by the Center for Disease Control (CDC) as a category B biological threat agent. The current emphasis on biological defense and continuing epidemicity of VEE have therefore renewed interest in development of a vaccine to prevent VEE infections. In addition, recent advancements in molecular biology of VEE have established the feasibility of genetic manipulation of this virus for use as viral vaccine vectors.

II. VEE Biology

i. VEE Structure and Organization

VEE is a spherical virus of about 70nm in diameter with icosaheral T = 4 symmetry. Its positive-sense, single-stranded infectious RNA genome of about 11400 nucleotides is encapsidated in an icosahedral protein shell composed of 240 copies of a

single species of protein (the capsid protein of 30-33 kilodaltons) and enveloped by a lipid bilayer derived from the host-cell plasma membrane. The outer surface of the virus is composed of flower-like projections of E1 and E2 glycoprotein heterodimers arranged as 80 trimers in the form of spikes (Figure 1.1a) (116, 165).

The VEE genome is arranged in two major regions: the 5' two thirds of the genome which encodes the nonstructural proteins (NsPs), NsP1, NsP2, NsP3 and NsP4, according to their order in the genome, and the 3' one third encoding five structural proteins (Capsid (C), E1, E2, and E3 glycoproteins, 6K protein) (Figure 1.1b) (156). The NsPs are required for transcription and replication of the RNA genome. The 5' terminus is capped with a 7-methylguanosine, and the 3' terminus is polyadenylated. Downstream of the non-structural genes and before the coding sequence of the structural genes lies a 26S subgenomic promoter which controls the synthesis of the subgenomic mRNA.

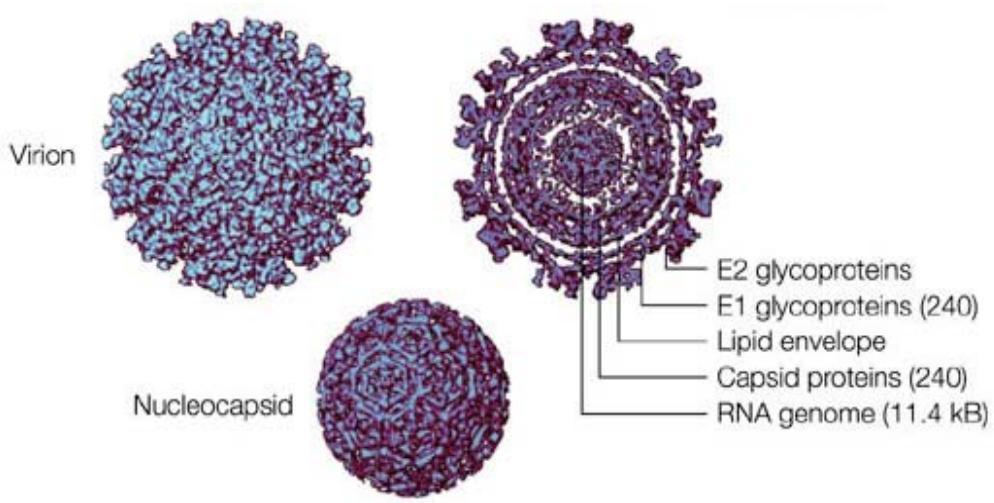
ii. Virus Life Cycle

Attachment and Entry: Binding of virus to the cell surface and entry into the cell is a multistep process that is dependent on virus glycoproteins E1 and E2, cell-surface molecules, low pH in the endosome, and fusion of membrane lipids. VEE, like many other alphaviruses, has a wide host range and replicates in a variety of different species, as well as in many different cell types. The wide host range is due to the ability of the virus to use several different receptors singly or in combination for binding. Some of the identified receptors include, major histocompatibility complex (MHC) class I molecule, the high affinity laminin receptor, and heparin sulfate (30, 77, 92, 167). The viral glycoprotein E2 is the protein that interacts with these receptors.

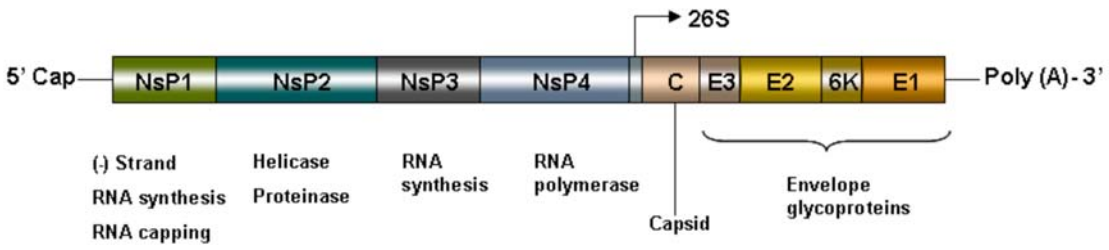
Figure 1.1a: Venezuelan equine encephalitis virus structural arrangement (116, 165).

Figure 1.1b: Venezuelan equine encephalitis virus genome organization. Adapted from Paredes and Weaver 2001.

A


Virion
Nucleocapsid
E2 glycoproteins
E1 glycoproteins (240)
Lipid envelope
Capsid proteins (240)
RNA genome (11.4 kB)

B


26S
5' Cap
NsP1
NsP2
NsP3
NsP4
C
E3
E2
6K
E1
Poly (A)-3'
(-) Strand
RNA synthesis
RNA capping
Helicase
Proteinase
RNA
synthesis
RNA
polymerase
Capsid
Envelope
glycoproteins

Entry into the cell after initial binding requires endocytosis followed by a conformational change in the trimer of E1-E2 heterodimers that is induced by exposure to low pH in the endosome (75, 169). E1 dissociates from E2 and forms E1 trimers, the fusion-active form of the protein (162, 163). Fusion of the virus with the endosomal membrane leads to the release of the nucleocapsid into the cytoplasm and is followed by an uncoating event that permits access of ribosomes to genomic RNA for initiation of translation.

Viral Transcription, Translation, Replication and Assembly: Translation of the virion RNA is initiated at a single AUG near the 5' terminus of the RNA. It proceeds uninterrupted for two thirds of the mRNA until encountering three termination codons located just downstream of the start of the sequences corresponding to the subgenomic RNA. The resulting polyprotein (P1234) serves as both a replicase and a transcriptase. It is cleaved into four distinct polypeptides; NsP1, NsP2, NsP3, NsP4. The protease responsible for these cleavages is located in the C-terminal domain of NsP2 (47, 73, 154). VEE has an opal termination codon a few codons before the NsP4 gene, and NsP4 is produced by read through of the opal codon followed by proteolytic cleavage (155).

A number of activities must be carried out by the NsPs in their role as replicative enzymes. The functions of each polypeptide were predicted through isolation, characterization, and mapping of temperature-sensitive (*ts*) mutants defective in viral RNA synthesis at the nonpermissive temperature as well as by gene-sequence homologies with known proteins (26, 27, 71, 72, 156). NsP1 has both guanine-7-methyltransferase and guanyltransferase activities, has been implicated in minus-strand RNA synthesis, and is involved in the association of viral replicase with membranes (3,

4, 72, 105, 142, 164). NsP2 is involved in regulation of minus-strand RNA synthesis, initiation of 26S subgenomic RNA synthesis, and proteolytic processing of the NsPs. It also possesses RNA helicase activity (47, 73, 154). NsP2 is found associated with the nuclear fraction of virus infected BHK-21 cells, although its function there is unknown (10, 122). NsP3: is required in some capacity for RNA synthesis, as evidenced by the isolation of a *ts* mutant defective in RNA synthesis at 40˚C, but its specific role remains unknown (72). NsP4 functions as the viral RNA polymerase (156).

RNA replication occurs via synthesis of a full-length minus strand intermediate that is used as a template for synthesis of additional genome-length RNAs and for transcription of a plus-strand 26S subgenomic RNA from an internal promoter (Figure 1.2) (56, 156). This subgenomic RNA, which can accumulate to levels approaching 10^6 molecules per cell, is the mRNA for translation of the structural proteins (Figure 1.2) (56). The synthesis of minus, plus and subgenomic RNAs is temporally regulated via proteolytic processing of nonstructural polyprotein replicase components by NsP2 protease activity (Figure 1.3) (97, 156).

The protein complex that initiates minus strand RNA synthesis is composed of P123 and NsP4. This complex is also able to synthesize plus strands, but inefficiently. Efficient synthesis of plus strand genomic and subgenomic RNAs occurs after trans-cleavage at the 1/2 and 2/3 sites of the polyprotein; the latter cleavage results in the shut-off of minus strand synthesis. Host factors (hf) are thought to be involved in these reactions (Figure 1.3) (97).

Figure 1.2: Alphavirus replication cycle. The sequence in the nonstructural region important for encapsidation is indicated by a solid box. The start site for subgenomic mRNA transcription on the (-) strand genomic-length RNA template is indicated by an arrow. Translation initiation and termination are indicated by triangles and diamonds, respectively. Adapted from Rice and Frolov 1996.

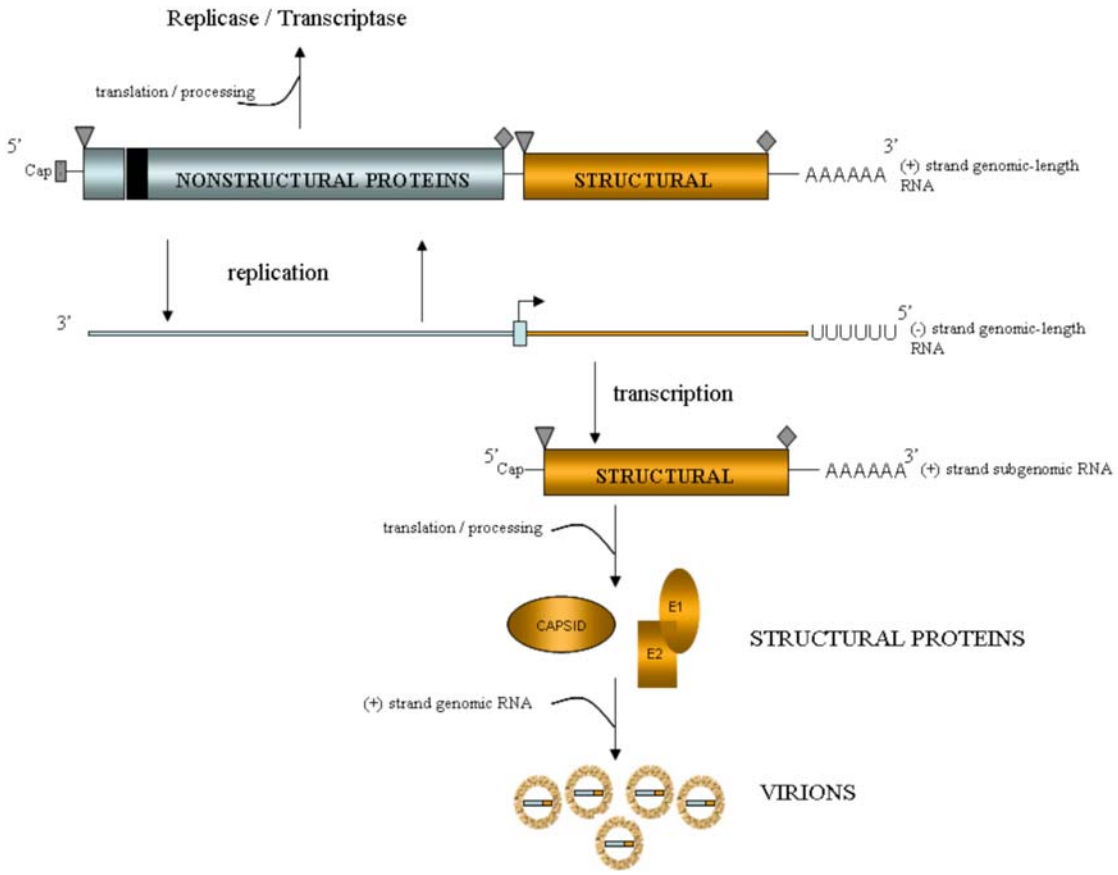
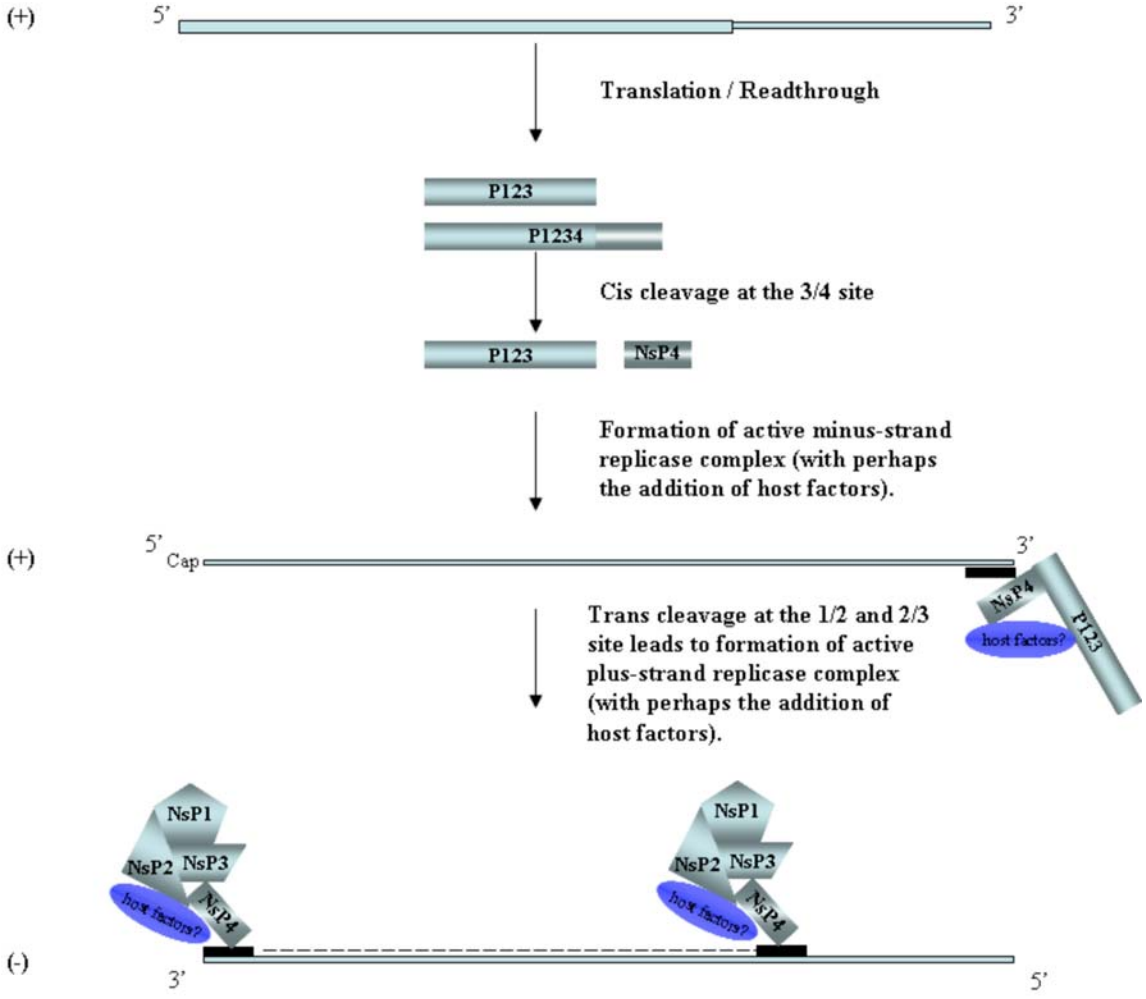

Replicase / Transcriptase
translation / processing
5'
Cap
NONSTRUCTURAL PROTEINS
STRUCTURAL
AAAAAA
3'
(+) strand genomic-length RNA
replication
3'
UUUUUU
5'
(-) strand genomic-length RNA
transcription
5'
Cap
STRUCTURAL
AAAAAA
3'
(+) strand subgenomic RNA
translation / processing
CAPSID
E1
E2
STRUCTURAL PROTEINS
(+) strand genomic RNA
VIRIONS

Figure 1.3: Schematic representation of a model for the temporal regulation of minus- and plus-strand RNA synthesis. Formation of replicase complexes specific for the synthesis of specific RNA species. Adapted from Lemm and Rice 1994.


(+)
5'
3'
Translation / Readthrough
P123
P1234
Cis cleavage at the 3/4 site
P123
NsP4
Formation of active minus-strand replicase complex (with perhaps the addition of host factors).
(+)
5'
Cap
3'
NsP4
host factors?
P123
Trans cleavage at the 1/2 and 2/3 site leads to formation of active plus-strand replicase complex (with perhaps the addition of host factors).
NsP1
NsP2
NsP3
NsP4
host factors?
NsP1
NsP2
NsP3
NsP4
host factors?
(-)
3'
5'

The structural proteins are translated from the subgenomic RNA early (2-3 hours after infection) in the replication cycle, as soon as the mRNA is formed. The resulting polyprotein (NH2-C-E3-E2-6K-COOH) is processed co- and post-translationally to produce the mature products (56). Cleavage at the C-E3 site is mediated by a chymotrypsin-like protease activity residing in the C-terminal portion of the C protein. E3 and E2 are initially made as a precursor (called PE2 or P62) that is processed by a furin-like activity late during release of the virus from infected cells. Envelope glycoproteins E1 and PE2, separated by signal peptidase cleavages, form a heterodimer that migrates through the secretory pathway to the plasma membrane. In the cytoplasm, C-protein subunits complex with the genome RNA to form a nucleocapsid that matures by budding through the plasma membrane, acquiring a lipid bilayer envelope with embedded viral glycoproteins (56, 156).

III. Effects of VEE Infection on Host Cell

Alphaviruses including VEE are transmitted to vertebrate hosts by mosquitoes. In these invertebrate host cells, chronic persistent infection is established with no apparent deleterious consequences. However, in permissive vertebrate host cells, virus infection leads to rapid shut-off of host-cell protein synthesis, a takeover of the translational machinery by viral mRNAs, production of high titers of infectious virus, extensive cytopathic effects (CPE) characterized by cell rounding, shrinkage, and cytoplasmic blebbing, and eventual cell death within 12 – 24 hours. The expression of viral nonstructural proteins is necessary and sufficient to induce CPE, however, expression of

structural proteins clearly exacerbates its onset (57). In several cell types, death appears to occur via apoptosis (67).

IV. Host Innate Immune Responses to VEE

Type I (α/β) Interferon (IFN) is induced after VEE infection of animal or human host (20, 60, 77, 84, 146, 160). Study of *ts* mutants suggests that formation of dsRNA is the necessary step in replication for IFN induction because viruses with mutations in the protease domain of NsP2, which cannot process the nonstructural polyprotein and thus cannot initiate plus-strand synthesis, do not induce IFN (72, 103, 163). IFN appears rapidly and may not require virus replication (9, 84). *In vitro* production of IFN follows the initial release of virus from infected cells by 2 to 3 hours (78). IFN is an important part of the host response to VEE infection. Animals can be protected from lethal infection if treated with IFN or IFN-inducers before or soon after infection (53, 84, 145, 158, 172). Animals unable to respond to IFN due to deletions of the IFN receptor or of crucial IFN signaling molecules may develop fatal infections even with avirulent virus strains (29, 65, 152). IFN acts primarily to limit virus replication early, during the time the specific immune response is being induced. Treatment of cells with IFN inhibits VEE replication (46, 84, 107, 109), but the mechanism by which this occurs, and therefore the IFN-induced host responses important for control of replication, are not known.

V. VEE as Viral Vectors – Adaptations and Applications

The original creation of an alphaviral self-replicating and packaging-deficient expression vector by Xiong et al. (1989) was the starting point for the rapid development

of various alphavirus vectors (174). Xiong et al. modified an infectious Sindbis (SIN) virus by replacing the structural protein gene region with the chloramphenicol acetyltransferase (CAT) gene. In order to package the replicon, or self-replicating RNA into particles, a helper SIN virus was used to supply the structural proteins. Moreover, by introducing a mutation in the non-structural gene region of the replicon that rendered it temperature-sensitive, Xiong et al. highlighted the promising potential of the system (174).

Full-length infectious vectors: A full-length infectious vector for Semliki Forest virus (SFV) was created in 1991 (98). Later, SIN and VEE were each engineered to contain a second subgenomic promoter upstream or downstream of the complete structural protein coding region, allowing high level expression of heterologous genes.

VEE replicon vectors: In these self-replicating vectors, the segment of the genome encoding the viral structural proteins has been replaced by a multiple cloning site. They retain the entire non-structural protein coding region as well as the natural subgenomic promoter. Packaged alphavirus-like particles are produced by cotransfection of *in vitro*-transcribed replicon RNA and a helper RNA encoding the structural proteins (22, 99). Productive replication and high level expression of foreign genes can be initiated either by transfection of the genomic RNA into the cytoplasm of the cell or by its infection with packaged alphavirus-like particles. The system is self limiting because helper RNAs, which lack the packaging signal, are not encapsidated. Thus, replicons are single-cycle vectors incapable of spreading from infected to non-infected cells. To reduce the risk of recombination to produce replication-competent viruses, the structural genes are split

onto two separate helper vectors (55, 149). Another approach renders the system conditional by necessitating proteolytic activation of the glycoproteins (13).

In vitro and in vivo applications: VEE and other alphavirus vectors are used in basic research for structural and kinetic studies of proteins because they are capable of robust protein expression. Intracellular, secreted and membrane proteins as well as receptors have been successfully produced using alphavirus replicon expression systems (100).

Vaccine vectors: Inherent features of VEE are the bases for its consideration as a formidable vaccine delivery vector. These features include high level expression of heterologous genes, tropism for lymphoid tissues, induction of mucosal as well as protective immunity, general lack of pre-existing immunity to VEE in the population, and the absence of risk of recombination into host cell genome (34, 42, 43, 64, 85, 89, 128). The ability of the virus to deliver 'danger signals' to the innate immune system may account in part for its immunogenicity (96).

For this thesis project, all immunizations utilized VEE replicon particles (VRPs) encoding various transgenes. VRPs were produced by cotransfection of Baby Hamster Kidney (BHK-21) cells with replicon RNA encoding the gene of interest (GOI) and two defective helper RNAs encoding the capsid and envelope glycoproteins of VEE as shown in Figure 1.4.

Wild-type and modified VEE replicons used in immunogenicity experiments in this thesis encoded the envelope glycoproteins of either human immunodeficiency virus type-1 (HIV-1) or Henipaviruses. Section VI and VII below provide some relevant

background information on HIV-1 vaccinology and Henipavirus (structure and envelope glycoproteins) respectively.

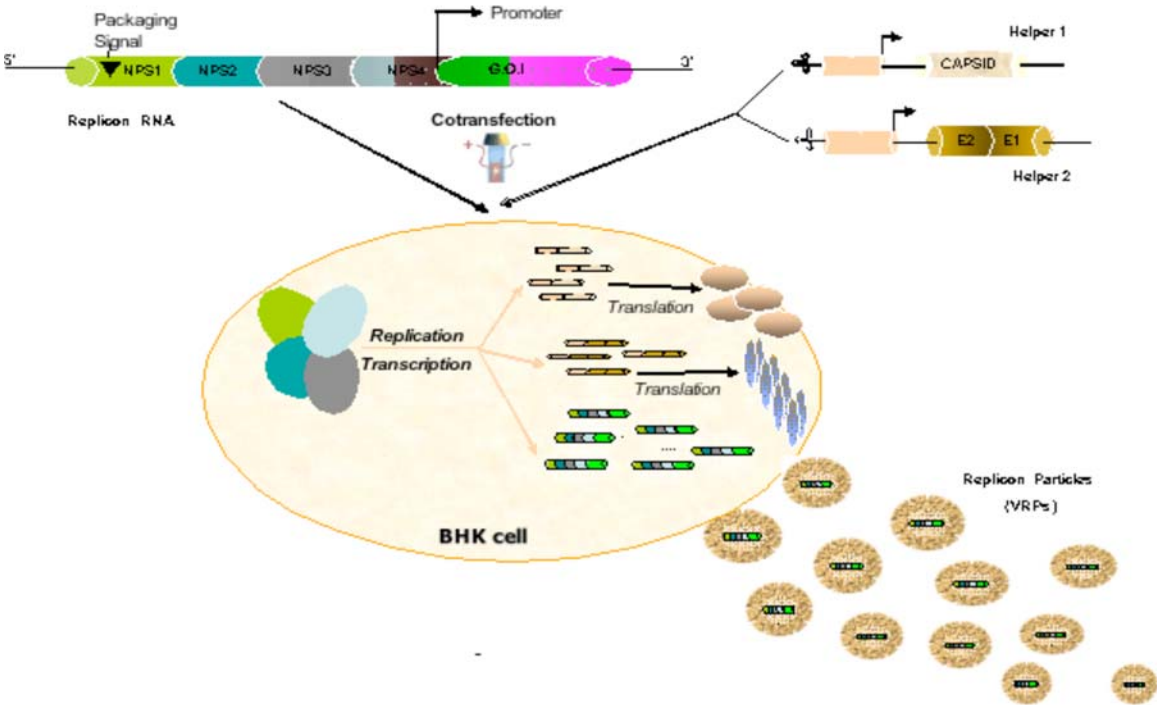
VI. HIV-1 Vaccinology

The spread of the HIV-1 epidemic in both the industrialized countries and the developing world provides compelling evidence for the need for an AIDS vaccine. More than 30 million people worldwide are currently infected with HIV-1, and more than 3 million new infections occur every year (25). Although the advent of highly effective antiretroviral therapy has resulted in significant increases in survival for HIV-1-infected individuals (69), the impact of combination antiretroviral therapy will be largely confined to the industrialized world. Historically, vaccines have been effective for control of infectious diseases and would be needed to curtail the HIV-1 epidemic.

Ideally, an HIV-1 vaccine should be efficacious in preventing transmission by mucosal and parenteral routes, should require a single low cost vaccination, be highly stable for facilitation of mass immunization campaigns, and should protect against infection with diverse viral isolates.

Realistically, given the scope of the AIDS pandemic, even an imperfect vaccine might result in a significant impact in AIDS transmission and diseases. However, a partially effective HIV-1vaccine that increased risk-taking behavior might paradoxically increase HIV-1 transmission. Vaccine induced protection against HIV-1 disease could be achieved by either complete protection from immunity (sterile immunity), or clearance of virus and infected cells (abortive infection), or persistent infection without disease (110).

Figure 1.4: Schematic of the process of VEE replicon particles production.


Packaging Signal
Promoter
5'
NPS1
NPS2
NPS3
NPS4
G.O.I
3'
Replicon RNA
Cotransfection
Helper 1
CAPSID
E2
E1
Helper 2
Translation
Replication
Transcription
Translation
BHK cell
Replicon Particles
(VRPs)

Humoral immune responses: The HIV-1 Env is initially synthesized as a precursor (gp160) that is subsequently cleaved to yield an extracellular domain (gp120) that is noncovalently associated with a transmembrane protein (gp41). Several features of the HIV-1 Env limit its ability to be neutralized by antibodies. gp120 is one of the most heavily glycosylated proteins identified to date, and these N-linked glycosylation sites are believed to limit the ability of antibodies to bind to gp120 (28, 120). In addition, the HIV-1 Env exists as an oligomer of three gp120-gp41 molecules. Oligomerization of the HIV-1 Env blocks the ability of antibodies that bind to epitopes present on the monomeric forms of Env to bind to the oligomeric form. (28, 120) The majority of antibodies present in HIV-1-infected people recognize monomeric gp120 and unprocessed gp160 rather than the mature oligomeric protein, (28). Antibodies able to neutralize primary HIV-1 strains generally arise between 2 and 6 months following primary infection, after the development of HIV-1 specific CTL activity and virus-binding antibodies (90, 106). Early neutralizing antibodies tend to be of low titer, are not present in all patients, and are only able to neutralize a limited range of isolates. Even following many years of HIV-1 infection, most infected subjects mount only weak neutralizing antibody responses against primary HIV-1 isolates (28, 120). However, there is growing evidence to support the role of neutralizing antibodies in protection which include: the ability of some antibodies to neutralize a broad range of HIV-1 isolates, including primary isolates (28, 120), and the correlation of high antibody titers induced by live attenuated SIV strains with protection (173).

Cellular immune response: Cytotoxic T lymphocytes (CTL) are a major host defense mechanism against viral infection (181). Although both CD4+ and CD8+ CTL

are the dominant effector cells responsible for defending the host against viral infection (181), CD8+ CTL recognize and lyse virus-infected cells following binding of the T-cell receptor to a viral peptide presented by a class I HLA molecule on the infected cell. The HLA type of an individual thus plays a major role in determining whether an individual will generate a CTL response to a given epitope. Presentation of viral proteins to CD8+ CTL generally requires synthesis of antigen within the cell. Most HIV-1-infected individuals develop a relatively strong virus-specifc CD8+ CTL response, as measured by a variety of *in vitro* assays (137). Observations that support the conclusion that HIV-1-specific CTL responses play a role in controlling viral replication *in vivo* include the ability of HIV-1-specifc CTL to select for the evolution of escape mutants (15), and to inhibit replication *in vitro* (175). Nonetheless, HIV-1 replication continues to occur at relatively high rates *in vivo*, despite the presence of a vigorous HIV-1-specific CTL response (86). CD4+ T helper cells play a critical role in regulating production of antibodies, induction and maintenance of CTL responses, and activation of macrophages and natural killer cells (1). CD4+ T helper function is typically measured by measuring T cell proliferation following incubation with viral antigens. Most HIV-1-infected subjects have weak or undetectable proliferative responses to HIV-1 antigens (143). However, relatively vigorous HIV-1 specific CD4+ T helper responses have been reported in long-term nonprogressors and subjects who received potent antiretroviral therapy early in the course of primary infection (136).

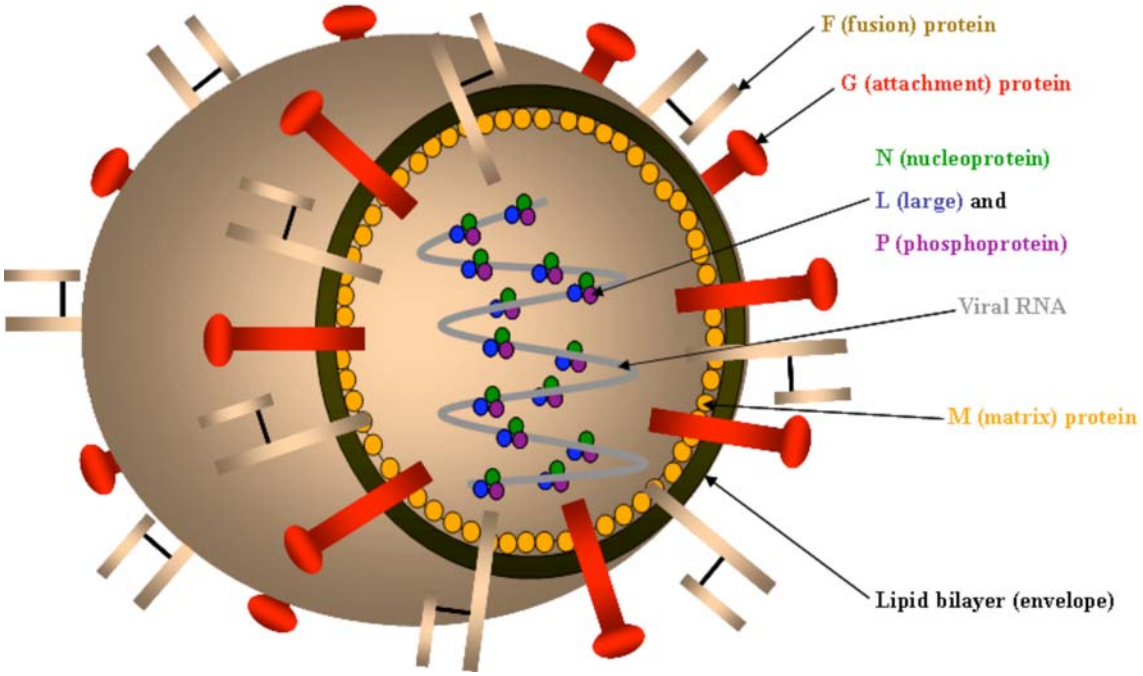
Obstacles to vaccine development: Sequence variation. Variation in HIV-1 arises both as a result of mutations introduced by the error-prone reverse transcriptase and by recombination between different viral strains (135). Based on phylogenetic analysis of

HIV-1 nucleotide and amino acid sequences, HIV-1 isolates have been grouped into 9 different major (M) subtypes (designated A through I) and a tenth outlier (O) (81). Because a significant proportion of HIV-1-specific neutralizing antibodies (62) and CTL (32), are type-specific, this sequence diversity has fostered efforts to induce broadly reactive immune responses or utilize multivalent vaccines. *Protective immunity*. Immunological correlates of protection remain unknown. A variety of immune responses have been described in HIV-1-exposed uninfected individuals, including proliferative responses (39), CTL responses (125), and mucosal HIV-1-specific immune responses (104), but it is not clear, however, if these immune responses are necessarily responsible for preventing infection or whether they merely reflect a prior abortive infection. *Latency*. Like other retroviruses, HIV-1 integrates into host genome (< 1%) and can be reactivated even following prolonged suppression by potent antiretroviral therapy (38). *Transmission*. HIV-1 is predominantly transmitted by mucosal routes yet our knowledge of mucosal infection events and mucosal immunity are limited.

VII. Henipaviruses

Introduction: Henipavirus is a genus of the family *Paramyxoviridae*, order *Mononegavirales* containing two members, Hendra virus (HeV) and Nipah virus (NiV). The henipaviruses are naturally harbored by Pteropid fruit bats (flying foxes) and are characterized by a large genome, a wide host range and their recent emergence as zoonotic pathogens capable of causing illness and death in domestic animals and humans. Henipaviruses are pleomorphic ranging in size from 40 to 600nm in diameter (83). They posses a lipid membrane overlying a shell of viral matrix protein. At the core is a single

Figure 1.5: : A schematic representation of henipavirus structure Adapted from Wikipedia image (171).


F (fusion) protein
G (attachment) protein
N (nucleoprotein)
L (large) and
P (phosphoprotein)
Viral RNA
M (matrix) protein
Lipid bilayer (envelope)

helical strand of genomic RNA tightly bound to N (nucleocapsid) protein and associated with the L (large) and P (phosphoprotein) proteins which provide RNA polymerase activity during replication. Embedded within the lipid membrane are spikes of F (fusion) protein trimers and G (attachment) protein tetramers (83). The function of the G-protein is to attach the virus to the surface of the host cell via ephrin B2, a highly conserved protein present in many mammals (14). The F protein fuses the viral membrane with the host cell membrane, releasing the virion contents into the cell. It also causes infected cells to fuse with neighboring cells to form large, multinucleated syncytia.

Henipavirus F and G glycoproteins: The G glycoproteins of henipaviruses are type II membrane glycoproteins consisting of a cytoplasmic tail, a transmembrane anchor region, a stalk and globular head which is composed of six propeller-shaped protein sheets (41, 94). Its globular head retains the propeller shape predicted for members of the family, and the location of neutralizing epitopes resembles that observed for other members of the *Paramyxovirinae* subfamily (50, 170, 176). The F glycoproteins are type I membrane proteins, and become biologically active when the F_0 precursor is cleaved to two disulphide-linked subunits, F_1 and F_2 by cathepsin L (50, 114, 141). They are also class I fusion proteins with two α-helical domains, referred to as heptad repeats, and are involved in the formation of a trimer-of-hairpins structure or six-helix bundle during or immediately following fusion (82, 93, 148). Peptide sequences that correspond to either heptad repeat of the F protein has been shown to inhibit both fusion (18, 93) and live virus infection *in vitro* (18). To date, efforts to develop protective vaccines against the henipaviruses utilize the envelope glycoproteins, based on similar successes in related

mumps and measles viruses (66, 68, 108, 115, 167). More introductory information of the henipaviruses can be found in Chapter 5.

BACKGROUND, SIGNIFICANCE AND AIMS OF THESIS

Expression of conformationally intact native proteins of human viruses, *in vivo*, is paramount in the elicitation of potent immunological responses to emerging infectious viral diseases. Substantial efforts have been expended on the development and application of *in vivo* expression systems that may be capable of producing such proteins in native confirmation. Among a plethora of viruses engineered to serve this purpose, alphavirus replicon systems have emerged as very successful vectors for *in vivo* expression of foreign viral genes for immunization against viral infections (30, 31, 44, 48, 76, 87, 127, 128). Model viruses studied include Influenza, Ebola, and the Human Immunodeficiency Viruses. Enveloped glycoproteins of each of these viruses have been expressed at high levels using VEE replicons, and have been delivered *in vivo* in VRP for immunization (30, 31, 44, 48, 76, 127, 128). In each case, the VRP immunization resulted in induction of neutralizing antibodies that protected animals against experimental challenge infection.

A number of studies conducted previously in our laboratory have established that immunization of mice and rhesus monkeys with HIV-1 envelope glycoprotein (Env) from a particular strain of HIV-1, designated R2, can induce antibodies that neutralize multiple strains of HIV-1 of diverse subtypes, including strains that are typical, neutralization resistant primary isolates (48, 129, 132, 177). This particular Env is from a naturally

occurring, CD4-independent strain of HIV-1. The antibodies induced in one previous non-human primate immunization study neutralized most, but not all strains of HIV-1 tested, and neutralized only one of three strains of pathogenic Simian-Human Immunodeficiency (SHIV). Only monkeys with the highest titers of neutralizing antibodies against the SHIV challenge virus were fully protected against intravenous challenge (129). To achieve effective protection against a broad selection of primary strains of HIV-1, it will undoubtedly be necessary to obtain higher, more potent neutralizing antibodies that cross-react with many strains of virus.

The VEE replicon system has been of interest for use in these HIV-1 immunization studies, because it has been used with credible success to induce immune responses that protect against a number of virus infections. The limited potency of the responses we obtained was unusual compared to the responses that have been reported with VEE replicons expressing glycoproteins of other viruses. Of course, the limited potency of the response may relate to the nature of the HIV-1 Env. However, the limited potency of the responses could also be related to the dynamics of VEE replicon expression of HIV-1 Env *in vivo*. In mouse immunogenicity studies, optimal antibody responses where obtained only when replicon doses where administered in clusters at short intervals (i.e., every 3 days x 3 doses) (48). This immunization approach was used to extend the period of protein expression *in vivo*, since cells transduced by replicons generally die quickly due to cytopathic effect. Cells transfected with the replicons, or transduced with replicons by infection with replicon particles, become cytopathic and die within 24-48 hours. The transient expression of the protein that is characteristic of VEE

replicon expression may not be sufficient to result in sufficiently differentiated, high affinity antibody responses.

In this study, we have also explored the potential for the development of a modified VEE replicon that can be employed for optimizing and enhancing immunogenicity when used as a vaccine vector in protection against infection, including the Category C biodefense agents, HeV and NiV. HeV and NiV are important human pathogens that have emerged only recently. HeV and NiV are the prototype members of the new genus, *Henipavirus,* in the *Paramyxoviridae* family, and are also zoonotic BSL-4 priority pathogens. HeV was first identified in Australia in 1994 as the cause of fatal respiratory illness in horses, and was transmitted to humans through close contact with horses. NiV was first described in 1998 during an outbreak in Malaysia in which several hundred individuals were infected, with nearly a 40% mortality rate. Both NiV and HeV are of particular concern for biodefense as they are easily grown in cell culture or embryonated eggs, are highly infectious and pathogenic, are transmitted easily via the respiratory tract, and can infect and be transmitted to humans from livestock. Being newly described, there is limited knowledge about these viruses, particularly their antigenic structure, and development of a vaccine that can induce potent neutralizing antibodies is a current priority. A collaborator in these studies, Dr. Christopher C. Broder, currently studies the biology of these viruses, describing their membrane fusion activity and developing experimental systems to explore the structure and function of their envelope glycoproteins.

Accordingly, the specific aims of this thesis project are:

1. Develop and characterize an attenuated or non-cytopathic alphavirus replicon that can express the HIV-1 Env gene for sustained periods *in vitro* and *in vivo*.

2. Determine the relative effectiveness for neutralizing antibody induction of immunization regimes incorporating singly or in combinations, wild-type and non-cytopathic alphavirus replicons.

3. Determine the effectiveness of wild-type and non-cytopathic alphavirus replicons for induction of antibodies that neutralize Hendra and Nipah Viruses.

CHAPTER 2: MATERIALS AND METHODS

I. Cell cultures

Baby Hamster Kidney cell line (BHK-21) (ATCC, Manassas, VA.), Human Embryonic Kidney (HEK) 293T cell line (ATCC, Manassas, VA.), and Human Osteosarcoma (HOS)-CD4-CCR5 cell line (NIH ARRRP; originally submitted by N. Landau) used in this study were maintained in Dulbecco's minimal essential medium (Gibco) supplemented with 10% fetal bovine serum, L-glutamine, penicillin-streptomycin (Gibco) and tylosin (Sigma). HOS culture medium in addition contained puromycin. HeLa (ATCC CCL 2) cell line was maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD.) supplemented with 10% cosmic calf serum (HyClone, Logan, UT.) and L-glutamine (DMEM-10). PCI 13 cells were maintained in DMEM-10 supplemented with 10 nM HEPES (Quality Biologicals). All cell cultures were maintained at 37˚C in humidified 5% CO_2 atmosphere.

II. VEE Replicon constructs

The VEE constructs pRepX (VEE replicon vector) and pCV have been previously described (128). They were kindly provided by J. Smith, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md. The pGPm has been previously described (48). It is a modified version of the pGP (128) with two back mutations E1 (272A/T) and E2 (209E/K) to facilitate lymphoid trafficking of replicon particles (48). All PCR amplified genes from donor plasmids that were cloned into pRepX, were amplified by primers designed to introduce a ClaI recognition sequence followed by a 16-nucleotide VEE promoter sequence at the 5' end of the gene, and a ClaI recognition sequence at the 3' end (48, 128). All PCR amplification reactions were performed using rTth DNA

polymerase (Applied Biosystems, Foster City, CA.). pRepX-GFP (V-GFP) was constructed by ligating a ClaI restriction fragment of the green fluorescence protein (GFP) gene into a similarly digested pRepX. The GFP gene was PCR amplified from pEGFP-N1 (Clontech Laboratories, Inc., Ca.). Mutations were introduced into the NsP2 region of V-GFP by site-directed mutagenesis (Stratagene Quick Change) according to manufacturer's instructions. Proline (P) ^{713} was replaced by either leucine (L), threonine (T), alanine (A), arginine (R), or glycine (G). A double mutation was also performed A259P/K646D. All constructs with the R, G or PD (double mutant) mutations described above are designated starting with V(R), V(G), or V(PD) respectively. The pRepX-R2gp160 (also named V-R2gp160) and its corresponding R2 Env gene has been previously described (48, 132). V-R2gp140 was constructed by ligating a ClaI restriction product of $gp140_{R2}$ amplified from the vaccinia vector pMCO2 (33, 129) into a similarly digested pRepX.

The V-HeVF, V-HeVG, V-NiVF and V-NiVG were constructed as VEE replicon vectors encoding the corresponding fusion (F) and attachment (G) glycoprotein of HeV and NiV. The constructs were made by ClaI restriction cloning of PCR amplified coding sequences of the corresponding Env glycoprotein into pRepX. The original vaccinia vector, pMCO3T7 with the corresponding F and G coding sequences were kindly provided by Dr. Christopher C. Broder., Uniformed Services University of the Health Sciences (USUHS), Bethesda MD.

All modified VEE replicon constructs carrying a single or double mutation in the NsP2 region were generated by replacing an ApaI/NotI digested fragment of the appropriate mutant V-GFP with a similarly digested fragment from a wild-type VEE

replicon construct encoding the appropriate transgene. This enabled the replacement of GFP in the mutant replicon constructs with another heterologous gene, while avoiding similar repetitive mutagenesis that could introduce unwanted mutations.

III. Generation of packaged VEE Replicon Particles

The two-helper system developed by Pushko et al. was employed in the preparation of VRPs. Replicon particle preparation has previously been described (47). Replicon plasmids V-R2gp160, V(G)-R2gp160, V(PD)-R2gp160, V-R2gp140, V(G)-R2gp140, V-HeVF, V-HeVG, V(G)-HeVG, V-NiVF, and V-NiVG together with helper plasmids pGPm, and pCV were linearized with NotI and *in vivo* transcribed using T7 RNA polymerase (Ambion, Austin, TX.) according to manufacturer's instructions. The resulting transcripts of individual replicon constructs were separately combined with transcripts of pGPm and pCV, and each mixture was used to transfect $2x10^7$ BHK-21 cells by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA.). The cells were seeded into 75-cm^2 tissue culture flasks and fed with medium. Cells transfected with wild-type constructs were maintained at 37°C for 27 h in 5% CO_2 atmosphere. Those transfected with replicon constructs carrying a single or double NsP2 mutation were maintained at 35°C and 28°C respectively. Medium from each flask was harvested and then clarified by centrifugation at 10,000 rpm for 30 min at 4°C in a Beckman L5-5E ultracentrifuge. The clarified medium was transferred to a 35-ml centrifuge tube, underlayered with 5 ml of 20% sucrose in phosphate-buffered saline (PBS), and ultracentrifuged at 24,000 rpm for 3 h to pellet the particles. The medium and sucrose were removed, the pellet was covered with 0.5 ml Dulbecco's Phosphate-Buffered Saline

containing Ca^{2+} and Mg^{2+} (D-PBS) and 0.1% fetal bovine serum (FBS) at 4°C overnight and then scraped off into D-PBS, and aliquots were stored at -70°C until needed.

Infectivity of replicon particle preparations and infectious particle concentrations were determined by an immunofluorescence assay (IFA), as has been previously described (48). All HeV and NiV specific immune sera used in these experiments were kindly provided by Dr. Christopher C. Broder., USUHS, Bethesda MD. BHK-21 cells were seeded at a density of $2x10^4$/well into wells of 16-well LabTek tissue culture slides (Nalge Nunc International), incubated overnight at the appropriate temperature ranging from 28˚C to 37˚C in 5% CO_2 atmosphere. Wells were inoculated with 50 µl aliquots of serial 10-fold dilutions of replicon particle preparations in D-PBS with 0.1% FBS. Medium was then added in 150 µl aliquots. After 24 h, the cells were fixed with cold acetone at -20˚C for 20 min, air dried, and stored at -20˚C. For IFA, the cells were rehydrated in PBS with 0.1% bovine serum albumin (BSA), blocked with PBS containing 7.5% BSA for 15 min. Cells were probed with the appropriate anti-serum (either globulin fraction of human HIV-1 immune sera, soluble HeVG (sHeVG) specific rabbit immune sera, or NiVF/HeVF-specific rabbit anti-sera) or negative serum that was diluted 1:200 in PBS containing 0.1% BSA for 1 h at room temperature. The wells were washed twice and the assays were developed by using the corresponding goat anti-human, or goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.). The numbers of infected cells per well were determined by fluorescence microscopy, and the volume inoculated and dilution of replicon particles applied to the particular well were used to calculate the concentration of infectious particles in the starting inoculum. A safety test was performed on each

preparation to detect for replication competence. Confluent BHK-21 cell culture in six-well plates were inoculated with 1:10 dilutions of replicon particle suspensions, allowed to adsorb for 1 h, washed three times with PBS, fed with medium, and incubated at 37˚C for 24 h. The medium was harvested, filtered through 0.45μm-pore-size filters, and stored at -70˚C. After three serial blind passages, the medium was inoculated into wells of tissue culture slides for IFA.

IV. Detection of *in vitro* protein expression by VEE replicon constructs

Western immunoblot analysis was used to test for protein expression by the various replicon constructs. BHK-21 cells were either transfected with replicon construct as described above or infected with replicon particles encoding the gene of interest. After 24 to 72 h, medium was collected, spun down to remove cell debris, and Halt™ protease inhibitor cocktail (PIERCE, Rockford, IL.) was added. The cells were washed with cold PBS, lysed and cell extracts prepared using M-PER® Mammalian Protein Extraction Reagent (PIERCE, Rockford, IL.). Cell extracts were briefly centrifuged to remove cell debris and Halt™ protease inhibitor cocktail (PIERCE, Rockford, IL.) was added. Samples were prepared by boiling in sample buffer containing 2-mercaptoethanol. Proteins in samples of cell extracts and medium were resolved by sodium dodecyl sulfate 4-12% polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to nitrocellulose membrane the blot was probed with HIV-1 positive human serum, sHeVG rabbit antiserum, $NiVF_{cytoplasmic\ tail\ (cyt)}$-specific rabbit antiserum, or $HeVF_2$-specific rabbit antiserum as appropriate. The blot was then incubated with alkaline phosphate-conjugated goat anti-human (or anti-rabbit) IgG (Bio-Rad) and developed.

V. Measurement of immunogen-specific antibody response

Antibody response in serum was measured by enzyme-linked immunosorbent assay (ELISA) using the ELISA starter accessory kit (Bethyl Laboratories, Inc.). For detection of HIV-1 specific antibody responses, Nunc MaxiSorp C bottom well plates (Bethyl Laboratories, Inc.) were coated at 100µl (5ng)/well with R2gp120 or R2gp140 protein diluted in coating buffer (Sigma Chemical). For detection of HeV and NiV G-specific antibody responses, plates were coated at 50µl (50ng)/well with sHeVG or sNiVG protein (kindly provided by Dr. Christopher C. Broder, USUHS, Bethesda MD.) diluted in coating buffer. For detection of HeV and NiV F-specific responses, cell lysates of BHK-21 cells transfected 36 h prior with replicon construct encoding the respective F glycoprotein were used to coat plates after dilution in coating buffer. Coated plates were incubated overnight at 4˚C. After blocking with Postcoat solution (Sigma Chemical) for 1 h at room temperature, plates were washed, 100 µl of serially diluted mouse or rabbit sera in sample/conjugate diluent (Sigma Chemicals) were added to the wells and the plates were incubated at room temperature for 1h. Sera were run in duplicate. Positive and negative control sera were included in each assay. Reaction mixtures were further developed by adding 100 µl of biotinylated anti-mouse or –rabbit IgG and horseradish peroxidase streptavidin (Vector Laboratories Inc., CA.) both diluted at 1:5000 to each well. Plates were incubated at room temperature for 1 h, washed, and a 100-µl aliquot of TMB Peroxidase Substrate & Peroxidase Solution B (Kirkegaard & Perry) was added to each well. Plates were incubated in the dark at room temperature for 30 min. Color development was stopped by addition of 50 µl of 2 M H_2SO_4 to each well. The optical density at 450 nm was measured in a Bio-Rad Model 680 microplate reader. Linear

regression curves were plotted for each serum sample, and the titers calculated as the inverse of the highest serum dilution that produced an optical density twice that of the negative control serum.

VI. HIV-1 pseudotyped virus neutralization assays

The ability of immune mouse or rabbit sera to inhibit pseudotyped virus infection of HOS-CD4-CCR5 cells has been previously described (48, 95, 117-119, 130, 131, 178, 179). Results obtained using this assay has been shown to be comparable to those obtained from similar experiments using conventional virus neutralization assays (179).The pseudoviruses were prepared by co-transfection of 293T cells with plasmids pNL4-3.luc.E-R- (NIH ARRRP; originally submitted by N. Landau) (74) and HIV-1-R2gp160 plasmid (118). Following 18 h of incubation, medium was replaced with medium containing 0.1mM sodium butyrate (Sigma) and cells were further incubated for 24 h at 37˚C in 5% CO_2 atmosphere. Pseudovirus-containing medium was collected, centrifuged at 1700 rpm for 5 min to remove cell debris, and then passaged through 0.45μm pore size filters (Millipore, Bedford, MA.) prior to use in neutralization assays.

Pseudotyped virus neutralization assays were performed by preincubation of 25 μl of two-fold dilutions of mouse or rabbit sera with 25 μl of pseudovirus suspension for 1 h at 4˚C in wells of 96-well, white-walled, flat-bottomed tissue culture plates (Costar, Corning, NY.). 150 μl suspensions of $1x10^4$ HOS-CD4-CCR5 cells were added to all wells, plates were incubated at 37˚C in 5% CO_2 atmosphere for 3 days, then washed with PBS and lysed for 30 min in shaker with 15 μl of Luciferase Assay System cell lysis buffer (Promega, Madison, WI.). Next, Luciferase Assay System reporter lysis buffer

(Promega) was added, and luciferase activity was measured by using a MicroLumat *Plus* Luminometer (Wallac, Gaithersburg, MD.) Neutralization titers were determined based on relative luminescence units (RLU). Neutralization endpoint was calculated as the highest serum dilution at which mean luminescence from test samples were less than 50% of non-neutralized controls. All test sera were run in triplicates in at least two independent experiments.

VII. Generation of Henipavirus pseudovirions and Neutralization assays

Neutralization assays using HIV-1 pseudotyped viruses has been widely employed, they are highly reproducible and quantitative (48, 95, 117-119, 130, 131, 178, 179). The pseudotyped virus assay has established a framework for testing the potency of immune sera and monoclonal antibodies against biological safety Level 3 (BSL-3) and 4 (BSL-4) pathogens.

Recently, HeV and NiV pseudotyped virions have been successfully produced and characterized using pNL4-3.luc.E-R- (74) and pCAGG plasmids encoding HeV and NiV envelope glycoproteins (Khetawat and Broder, unpublished). This milestone has added a new tool for studying the structure and function of henipavirus envelopes as well as the characterization of immune sera and monoclonal antibodies against these BSL-4 agents in BSL-2 conditions.

HeV and NiV pseudovirions were prepared using an established protocol with slight modifications. Briefly, pseudoviruses were prepared by FuGENE® 6 Transfection Reagent (Roche)-mediated co-transfection of 60-80% confluent 293T cells in 25-cm^2 flask with plasmids pNL4-3.luc.E-R- and pCAGG-$Henipavirus_{envelope.}$ For homotypic

NiV and HeV pseudoviruses; pCAGG-NiVF and –NiVG or pCAGG-HeVF and –HeVG were used respectively. For heterotypic pseudovirus preparation, pCAGG-HeVF and –NiVG were used. Following 24 h of incubation, fresh medium was added and cells were further incubated for 24 h at 37˚C in 5% CO_2 atmosphere. Pseudovirus-containing medium was collected, centrifuged at 1700 rpm for 5 min to remove cell debris, and then passaged through 0.45μm pore size filters (Millipore, Bedford, MA.) prior to use in neutralization assays. Neutralization assay was performed as described above with 293T cells used as target cells. Positive and negative controls were included in each assay and serum samples were run in triplicates in at least two independent experiments. Neutralizing antibody titers were calculated as 90% inhibitory endpoints.

VIII. Cell fusion assays

NiV and HeV glycoprotein-mediated cell fusion reporter gene assay has previously been described and used extensively (14, 16-19). It is an adaptation of a previously described reporter gene assay based on gene expression using the recombinant vaccinia virus system (12, 112). In addition to expression of viral envelope glycoproteins and viral receptors on effector and target cell populations respectively, one cell population also expresses a T7 RNA polymerase while the other expresses a T7 promoter-driven E.coli *lacZ* cassette. β-galactosidase (β-Gal) is synthesized only in fused cells (24, 112). Briefly, vaccinia virus encoded proteins were produced by infecting cells at an MOI of 10 and incubating infected cells at 31˚C overnight (12). HeLa cells were infected with recombinant vaccinia viruses encoding F and G glycoproteins of either HeV or NiV, along with a recombinant vaccinia virus encoding T7 RNA polymerase (effector

cells). PCI (target cells) infected with the reporter vaccinia virus, vCB21R, encoding E. coli *lac*Z were mixed with effector cells in duplicate wells of a 96-well plate and incubated at 37˚C. The ratio of envelope glycoprotein expressing effector cells to target cells was 1:1 ($2x10^5$ total cells per well; 200µl total volume). Cytosine arabinoside (40 µg/ml) was added to the fusion reaction mixture to reduce nonspecific β-Gal production (12). For quantitative analysis, Nonidet P-40 was added (0.5% final) at 2.5 h, and aliquots of lysates were assayed for β-Gal at ambient temperature with the substrate chlorophenol red-D-galactopyranoside (Roche). For testing fusion inhibition by immune sera, serial dilutions of mouse sera were performed and added to target cell populations 30 min prior to the addition of effector cell population. Positive and negative controls were included in each assay and fusion results were calculated and expressed as rates of β-Gal activity (change in optical density at 570 nm per minute x 1,000) (112). The 50% inhibitory endpoints were determined by comparing the rates of β-Gal activity in test sample wells at each dilution to the rate of β-Gal activity in negative control wells. The highest dilution at which the test serum inhibited fusion to less than 50% of the control was considered the endpoint.

IX. Flow Cytometry

Wild-type and modified VEE replicon constructs encoding GFP were examined for their ability to induce cytopathogenicity in BHK-21 cells. Cell populations were transfected with replicon transcripts as described previously. At various time intervals, cells were harvested and stained with Annexin V-Phycoerythrin (Annexin V-PE) (BD Biosciences), a marker for apoptosis, and 7-Amino-actinomycin D (7-AAD) (BD

Biosciences), a marker for apoptotic and nonviable cells. Staining was performed in accordance with manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h post staining using the Beckman Coulter EPICS XL-MCL Cell Analyzer. Appropriate positive and negative controls were included in each experiment. Viable replicon-transfected cell populations were defined as GFP positive, Annexin V-PE and 7-AAD negative.

X. Mouse and rabbit immunization

Animals were used with approval of the Institutional Animal Care and Use Committee. Six- to eight-week-old female C3H/He mice (Jackson Laboratories) were used for immunogenicity studies. All mice were immunized by footpad inoculation of 50-µl suspensions of replicon particles. Mice were bled from the tail vein. Adult New Zealand White rabbits (Jackson Laboratories) were immunized by subcutaneous inoculation of 200-µl suspensions of replicon particles into the lateral aspects of the lower hind leg. Rabbits were bled from ear veins.

XI. Statistical analysis

For comparing animal responses among and between various immunization groups, a one-way ANOVA with Bonferroni's Multiple Comparison Test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA.

CHAPTER 3: GENERATION AND CHARACTERIZATION OF PROLONGED-EXPRESSION VARIANTS OF VENEZUELAN EQUINE ENCEPHALITIS REPLICONS

INTRODUCTION

Alphavirus replicons have been used successfully as vectors for *in vivo* expression of foreign viral genes for immunization against viral infections. Model viruses that have been studied include Influenza, Ebola, and Human Immunodeficiency Viruses. Envelope glycoproteins of each of these viruses have been expressed at high levels using replicons derived from Venezuelan Equine Encephalitis Virus (VEE), and have been delivered *in vivo* in replication-defective virus particles for immunization. Immunization of small animals and monkeys with VEE replicons expressing HIV-1 or SIV envelope and Gag proteins resulted in neutralizing antibody and other immune responses and substantial partial protection against virulent, intravenous, heterologous SIV challenge (30, 31, 44, 48, 87, 129). Nonetheless, substantial potential limitations do exist in relations to immunogenicity issues when using VEE replicons as vaccine vectors.

One of such concerns is rather paradoxical. High replicon replication and protein expression is associated with rapid development of cytopathic effects (CPE) in transduced cells within 24 - 48 hours. The cytopathicity is thought to be due mostly to effects of VEE non-structural proteins (NsPs) but is also contributed to structural proteins (57). It is likely that the duration of protein expression for orchestration of a sufficiently differentiated, high affinity antibody response is limited. This interpretation has been consistent with the experience of our lab with small animal HIV-1 immunogenicity studies, and with primate studies on SIV immunogenicity and challenge by Davis et al. (44, 48, 87). While the limited potency of the responses induced may relate to the nature of the HIV-1 Env, it could also be related to the dynamics of VEE replicon expression of HIV-1 Env *in vivo*. In fact, in mouse immunogenicty studies carried out in this

laboratory, optimal antibody responses were obtained when replicon doses where administered in clusters at short intervals (i.e., every 3 days x 3 doses) (48). This immunization was used to extend the period of protein expression *in vivo*, since cells transduced by replicons generally die quickly due to cytopathic effects as mentioned earlier.

The VEE replicon constructed by Pushko et al consists of a 5' two-thirds region encoding the non-structural protein genes (NsP1-NsP4) while the 3' end encodes the subgenomic structural genes (or transgene in the case of VEE vectors) under the control of an internal promoter. Upon transfection of replicon or transduction of replicon particles into cells, the NsPs are translated into a replicase polyprotein (P1234). RNA replication proceeds through a full-length minus-strand intermediate which serves as the template for synthesis of both full-length and subgenomic RNA. Synthesis of all three RNA species is highly regulated, with shutoff of minus-strand RNA synthesis by 3 to 4 h post-introduction into cell (139, 140). This temporal regulation is thought to occur through a proteolytic processing cascade orchestrated by the protease domain residing in the NsP2 region of P1234 (97, 147). This proteolytic activity is responsible for processing of P1234 by cis cleavage at the 3/4 site and trans cleavage at the 1/2 and 2/3 sites to yield four structural proteins: NsP1 NsP2, NsP3, and NsP4 (54, 156). Replicases that contain uncleaved NsP2-containing polyproteins (P123 or P23) are required for efficient minus-strand initiation, while complexes containing the four mature NsPs mediate subgenomic and genomic plus-strand synthesis but are defective for synthesis of minus-strand RNAs. The other members of the RNA replicase complex play vital roles for viral replicon function. NsP1 is required for capping and minus-strand RNA

synthesis, NsP3 is required in some capacity for RNA synthesis, and NsP4 functions as the viral RNA polymerase (4, 47, 71, 156).

A series of spontaneous mutations have been discovered in the NsPs, especially in NsP2 and NsP4 with profound effects on viral cytopathogenicity. In Sindbis virus (SIN) and Semliki Forest Virus (SFV) vectors, mutations in the NsP2 gene were shown to reduce cytopathogenicity (2, 49, 123). Most of the mutants had single changes at amino acid residue 726. Temperature sensitive (ts) mutations have been described for SIN and SFV (Semliki Forest Virus) in NsP2 and NsP4 genes (71, 72, 101). A study on replication-competent SFV also revealed that a point mutation (R649D) in the NsP2 nuclear localization signal confers a lower virulence in mice (51, 134). In addition, the introduction of known temperature sensitive mutations into NsP2 and NsP4 were shown to reduce the cytotoxicity of SFV vectors (101, 102). Furthermore, adaptive mutations in NsP genes in SIN and SFV vectors containing the puromycin or neomycin resistance gene, generated replication-persistent vectors that had either deletions or point mutations in NsP2 (54, 123).

In this study, we describe results of efforts to develop modified VEE replicons with attenuated, prolonged-expression phenotypes. This was accomplished by modifying the NsP2 gene of VEE to contain mutations that are known to reduce cytotoxicity, and enhance transgene expression in SFV and SIN. VEE replicons with such attributes may hold great potential for enhancing immunogenicity when used as vaccine vectors in studies requiring efficient and optimal antigen expression.

RESULTS

I. Development and characterization of modified VEE replicons

Attenuated VEE replicons that can persist in transduced BHK-21 cells and express for sustained periods of time may serve as attractive vaccine vectors. This hypothesis is based on the premise that the attenuated, prolonged-expression replicons would induce stronger immune responses *in vivo*, by increasing the duration of protein presentation to immune cells. Initial steps to test this hypothesis would require the generation of VEE replicons with mutations in replicase proteins that have been shown in other alphaviruses to encode a non-cytopathic phenotype in mammalian cell lines. The VEE replicon encoded by the plasmid, pRepX, contains sequences encoding the non-structural proteins of VEE, which constitute the viral replicase. Following the viral replicase are downstream sequences that encode the subgenomic promoter and a multiple cloning site in which the GFP gene has been introduced to serve as a reporter gene. Targeted mutations were introduced into the NsP2 region of pRepX, at highly conserved residues similar to those that have been previously reported to extend expression of genes encoded by Sindbis and Semliki Forest Viruses (51, 54, 100, 101). Initially, single mutations were made in the NsP2 gene using the Quick Change® XL site directed mutagenesis kit. Mutations were tyrosine (T) or leucine (L) substitutions for a conserved proline 713 in NsP2 resulting in NSP2-P713T (or V(T)-GFP) and NSP2-P713L (or V(L)-GFP) mutant replicons respectively (Figure 3.1).

Figure 3.1: Sequence alignments of NsP2 regions in which mutations were introduced are shown for VEE, SIN, SFV and pRepX (plasmid encoding the VEE replicon). Arrows indicate amino acid residues where mutations where were introduced; the mutations are indicated above the alignment for the double mutant VEE replicon and below the alignment for the single mutant VEE replicons. Since the length of NsP2 varies between SFV, SIN and VEE, codon numbering is indicated for each virus. The boxes highlight the conservativeness within the different viruses at the positions were mutations were introduced. NLS: denotes the nuclear localization signal sequence.

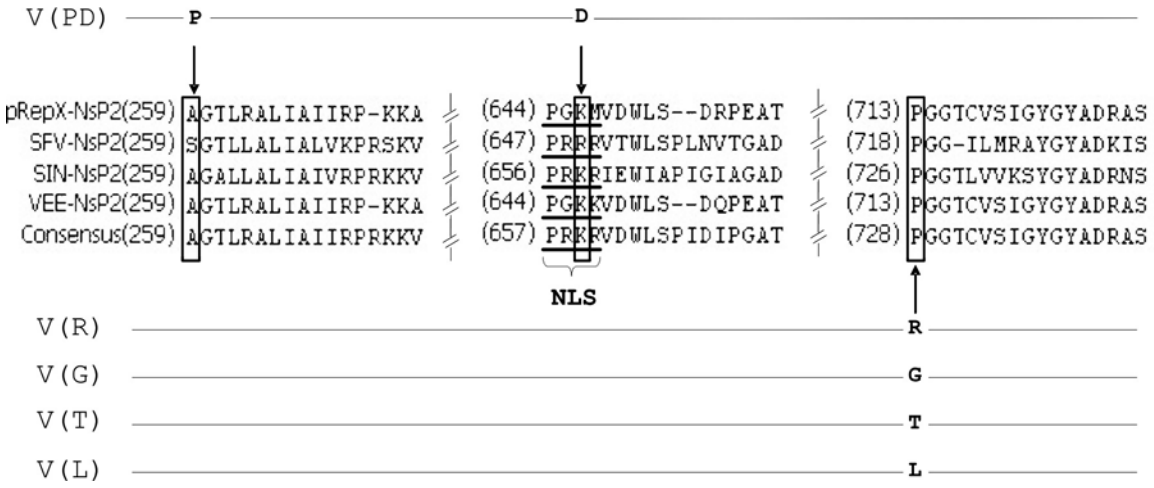
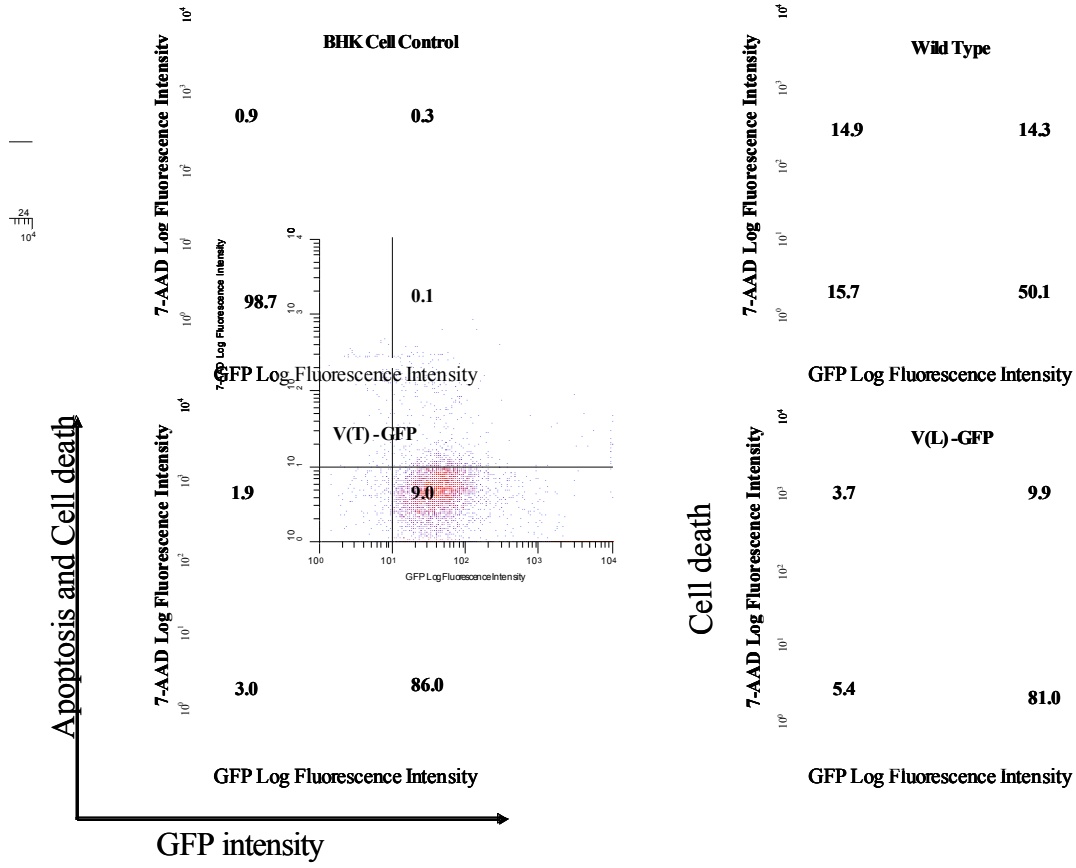

V(PD)
P
D
pRepX-NsP2(259) AGTLRALIAIIRP-KKA (644) PGKMVDWLS--DRPEAT (713) PGGTCVSIGYGYADRAS
SFV-NsP2(259) SGTLLALIALVKPRSKV (647) PRRRVTWLSPLNVTGAD (718) PGG-ILMRAYGYADKIS
SIN-NsP2(259) AGALLALIAIVRPRKKV (656) PRKRIEWIAPIGIAGAD (726) PGGTLVVKSYGYADRNS
VEE-NsP2(259) AGTLRALIAIIRP-KKA (644) PGKKVDWLS--DQPEAT (713) PGGTCVSIGYGYADRAS
Consensus(259) AGTLRALIAIIRPRKKV (657) PRKRVDWLSPIDIPGAT (728) PGGTCVSIGYGYADRAS
NLS
V(R)
R
V(G)
G
V(T)
T
V(L)
L

Figure 3.2: Flow cytometric analyses of apoptosis induction 48 hours after transfection of BHK-21 cells with wild-type, control or mutant VEE replicons expressing GFP.Mutations in replicon were tyrosine or leucine substitutions for proline 713 in NsP2. Cell populations were transfected with replicon transcripts. At 48h posttransfection, cells were harvested and stained with 7-Amino-Actinomycin (7-AAD), a marker for apoptotic and non-viable and nonviable cells. Cells were analyzed by flow cytometry within 1h post staining using the Beckman Coulter EPICS XL-MCL Cell Analyzer. Viable replicon-transfected cell populations were defined as GFP positive, 7-AAD negative Number in each gate represents the percentage of the total cell population present in that gate.


BHK Cell Control
7-AAD Log Fluorescence Intensity
0.9
0.3
98.7
0.1
V(T) -GFP
1.9
9.0
3.0
86.0
GFP Log Fluorescence Intensity
Wild Type
14.9
14.3
15.7
50.1
V(L) -GFP
3.7
9.9
5.4
81.0
Apoptosis and Cell death
GFP intensity
Cell death

Mutant clones together with wild-type replicon were transcribed to RNA. Upon transfection of mutant replicons V(T)-GFP and V(L)-GFP into BHK-21 cells, cytopathogenicity was delayed and GFP expression was prolonged.

As shown in Figure 3.2, a significant percentage of BHK-21 cells transfected with the wild-type replicon were apoptotic and displayed reduced GFP fluorescence after 48 h. In contrast, BHK-21 cells transfected with either of two mutant replicons remained more than 80% viable, with more than 90% of cells displaying clear GFP fluorescence. Subsequent NsP2 mutations introduced into the VEE vector included; three single mutations, V(A) (P713A) , V(R) (P713R), V(G)- (P713G), and a double mutation, V(PD) A259P/K646D (see Figure 3.1). The resulting transcripts were used to transfect BHK-21 cells and the expression profile and cytotoxicity phenotypes relative to the wild-type replicon were analyzed. Analyses were performed at various time points using fluorescent microscopy and flow cytometry.

Fluorescent microscopy revealed comparable GFP expression in viable cells by both wild-type and single mutants (V(A)-GFP, V(R)-GFP, and V(G)-GFP) 24 h posttransfection (Figure. 3.3). However, by 72 h posttransfection, cells transduced with the wild-type replicon displayed minimal GFP expression and significant cell rounding suggestive of apoptosis. In contrast, greater cell viability and sustained GFP expression were observed in cells transfected with either of three single mutants (Figure.3.3). In cells transected with A259P/K646D replicon, GFP expression was observed as long as 168 h posttransfection (Figure.3.3).

Figure 3.3: Expression profile of wild-type and variant VEE replicon constructs encoding GFP as a function of time. BHK-21 cells were electroporated with either wild-type or mutant replicon constructs. Cells were seeded into 75-cm^2 tissue culture flasks and maintained at 37˚C in 5% CO_2 atmosphere. At the indicated time points, photomicrographs of cells in a predetermined area of each flask were taken.

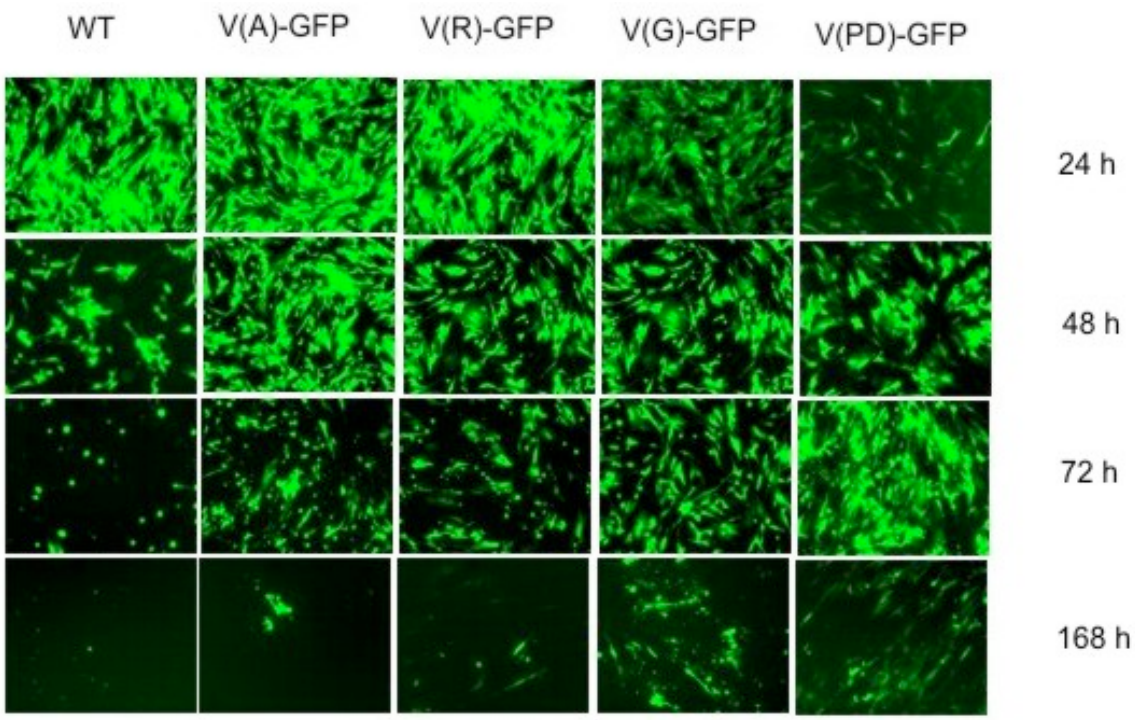
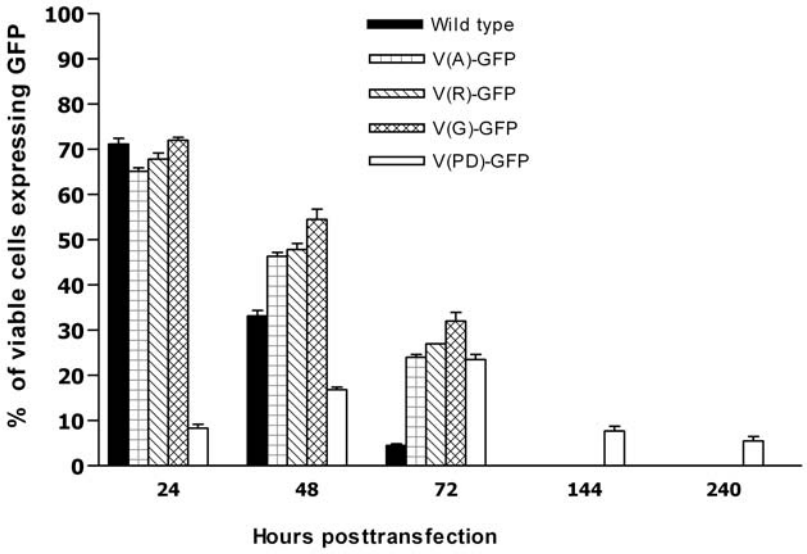

WT
V(A)-GFP
V(R)-GFP
V(G)-GFP
V(PD)-GFP
24 h
48 h
72 h
168 h

Figure 3.4: Relative cell viability and GFP expression in BHK-21 cells transfected with wild-type or mutant replicons. BHK-21 cells were electroporated with wild-type or variant VEE replicon RNA encoding GFP. At indicated time points posttransfection, the cells were analyzed by flow cytometry, and the proportion of viable GFP-expressing cells (GFP^+, $7\text{-}AAD^-$) was plotted. Data are the means of three independent electroporations ± SD. The background reading from mock-transfected BHK-21 cells was subtracted from all the samples.


Wild type
V(A)-GFP
V(R)-GFP
V(G)-GFP
V(PD)-GFP
% of viable cells expressing GFP
100
90
80
70
60
50
40
30
20
10
0
24
48
72
144
240
Hours posttransfection

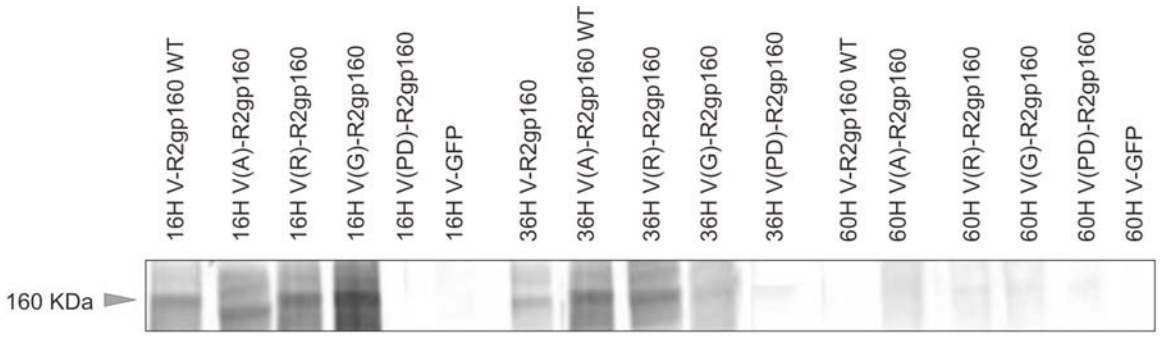
Upon flow cytometric analysis of the replicon transfected cells, the proportion of wild-type-transfected cells expressing GFP was markedly reduced 72 h posttransfection relative to the proportion of mutant replicon-transfected cells (Figure 3.4). The double mutant was able to mediate GFP expression up to 240 h posttransfection albeit at low levels (Figure 3.4).

II. Construction and characterization of mutant replicons encoding R2gp160

With the observed attenuated, prolonged-expression phenotype seen with the modified VEE replicons, we sought to determine whether the observed phenotype was encoded solely by the replicase mutation, irrespective of the expressed transgene. Thus, GFP was replaced by an HIV-1 Env, designated R2gp160, isolated from a patient whose sera possesses broadly cross neutralizing antibodies (130, 178). The reason for selecting R2gp160 as the replacement transgene was two fold. First, this laboratory has extensively characterized the R2 Env including its expression from VEE replicons (48, 130). Second, the use of this Env in future immunogenicity studies incorporating the modified replicons had been planned, since the experience in the laboratory has established an immunization regimen in small animals that can be used to reliably demonstrate immunogenicity to HIV-1 Env.

V-R2gp160 (wild-type), V(A)-R2gp160, V(G)-R2gp160, and V(PD)-R2gp160 were constructed by replacing the GFP reporter gene with R2gp160 in the wild-type, P713A, P713R, P713G, and A259P/K646D replicon templates, respectively. Next, BHK-21 cells were transfected with the corresponding replicon RNAs.

Figure 3.5: Expression of HIV-1 Env (R2gp160) by wild-type and variant VEE replicons. BHK-21 cells were electroporated with wild-type or mutant replicon RNA encoding R2gp160. At various time points posttransfection, cell lysates were prepared and resolved using 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred. Immunoblot was probed with HIV-1 positive human serum as described in Materials and Methods. V-GFP was included as a negative control.


16H V-R2gp160 WT
16H V(A)-R2gp160
16H V(R)-R2gp160
16H V(G)-R2gp160
16H V(PD)-R2gp160
16H V-GFP
36H V-R2gp160
36H V(A)-R2gp160 WT
36H V(R)-R2gp160
36H V(G)-R2gp160
36H V(PD)-R2gp160
60H V-R2gp160 WT
60H V(A)-R2gp160
60H V(R)-R2gp160
60H V(G)-R2gp160
60H V(PD)-R2gp160
60H V-GFP
160 KDa

Total cell lysates were collected at various time points and subjected to Western Blot analyses to determine the expression profile of each replicon. With the wild-type replicon, R2gp160 protein expression was detected at 16 h and 36 h posttransfection, but was undetected thereafter (Figure 3.5). In contrast, R2gp160 protein expression by V(R)-R2gp160 and V(G)-R2gp160 mutant replicons could still be detected 72 h posttransfection (Figure. 3.5). The double mutant replicon, V(PD)-R2gp160, manifested a delayed expression profile, with R2gp160 protein expression first detected after 36 h. There was no significant difference in expression intensity during the first 16-36 h between wild-type and mutant replicons except for the V(PD)-R2gp160 replicons which expressed at much lower levels. Overall, the R2gp160 expression profile of the modified VEE replicons was consistent with that seen with GFP expression.

III. Preparation of modified VEE replicon particles

The successful development of attenuated prolonged-expression VEE replicons allowed for their inclusion in immunization studies. These studies would assess the potential of modified vectors to enhance immunogenicity. Based on the cytotoxic effects and expression profiles of the mutant replicons, two single mutants, P713R and P713G, and the double mutant A259P/K646D were selected for further investigation. For use in future comparative immunogenicity studies, wild-type and selected modified VEE replicons would have to be packaged into replicon particles. This section describes the observations made following attempts to package the newly generated mutant replicons encoding various immunogenic transgenes.

The two-helper system developed by Pushko et al was used for construction of packaged replicon particles, as described in materials and methods. Infectivity of replicon particle preparations and infectious particle titers were determined by IFA, also described in Materials and Methods.

Initially, wild-type and modified replicon particles encoding the R2gp160 Env were prepared in parallel at 37˚C following the standard protocol. Titration of the replicon particles at the same temperature revealed approximately a ten-fold higher titer of wild-type particles, V-R2gp160, ~ 3-8 x 10^7 IU/ml, compared to particles incorporating the single mutants, V(R)-R2gp160 and V(G)-R2gp160 (~ 1-4 x 10^6 IU/ml) (See Table 3.1). No infectious particles were detected in preparations of the double mutant, V(PD)-R2gp160, at 37˚C. When the modified replicon particles were prepared at 35˚C, titers of V(R)-R2gp160 and V(G)-R2gp160 (~ 2-6 x 10^7 IU/ml) were comparable to wild-type particles (V-R2gp160) prepared at 37˚C (Table 3.1). V(PD)-R2gp160 titers were ~ 1-4 x 10^4 IU/ml when prepared at 35˚C. A 2 $\log_{10}$ increase in V(PD)-R2gp160 titers (~1-1.5 x 10^6 IU/ml) was observed when particles were prepared at 28˚C, although the titer was much lower than those seen with the single mutants (Table 3.1). Wild-type and modified replicon particles encoding henipavirus fusion (F) or attachment (G) glycoproteins were also prepared for use in immunogenicity studies (Chapter 5). In each case, the vectors exhibited similar temperature-dependent expression and packaging characteristics to those of R2gp160-encoding vectors harboring similar mutations. A summary of the characteristics of the various mutant replicons is shown in Table 3.1.

*Table 3.1: Comparison of wild-type and modified VEE vectors as a function of incubation temperature. * Titers were determined by an immunofluorescence assay method described in Materials and Methods. **, Transgene expression was determined based on: (a) flow cytometry measurements of GFP intensity in BHK-21 cell populations transfected with equal amounts of wild-type or mutant replicon RNA, (b) relative duration and intensity of GFP expression in BHK-21 cells transfected with equal amounts of wild-type or mutant replicon RNA as observed by fluorescent microscopy, (c) relative amounts of protein expressed by wild-type and mutant replicons in BHK-21 cells as measured by western blot. ***, The degree of cytotoxicity was assessed by using a cell viability probe, 7-AAD, in FACS analysis and by determination of cytopathic effects through microscopy. -, No detectable cytotoxicity; +/-, minimal delayed expression or cytotoxicity; +, relatively low expression or cytotoxicity; +++, high expression or cytotoxicity; nd, not determined; N/A, not applicable.*

Vector	Mutation	Titers obtained (IU/ml)*			Transgene expression**			Cytotoxicity***		
		28˚C	35˚C	37˚C	28˚C	35˚C	37˚C	28˚C	35˚C	37˚C
Wild type	N/A	nd	nd	3 - 8 x 10^7	nd	nd	+++	nd	nd	+++
V(R)	P713R	nd	2 - 6 x 10^7	1 - 4 x 10^6	nd	+++	+++	nd	+	+
V(G)	P713G	nd	2 - 6 x 10^7	1 - 4 x 10^6	nd	+++	+++	nd	+	+
V(PD)	S259P/K646D	1 - 1.5 x 10^6	1 - 4 x 10^4	—	+	+/-	+/-	+/-	+/-	+/-
V(L)	P713L	nd	nd	nd	nd	nd	+++	+	+	+
V(T)	P713T	nd	nd	nd	nd	nd	+++	+	+	+

DISCUSSION

Modified VEE vectors were developed that are capable of *in vitro* trangene expression for extended periods compared to wild-type vector. In four of the modified vectors; V(R), V(G), V(L), and V(T), the modification was accomplished in each case by a single substitution at amino acid residue 713 in the non-structural protein 2 gene (NsP2) (Figure 3.1). This substitution was at the c-terminal domain of NsP2 also known to encode its protease activity. In one case, a double mutation was introduced into NsP2, V(PD). The first mutation was at the N-terminal region and involved the substitution of alanine at residue 259 with proline. The second was made in the reputed nuclear localization signal of VEE NsP2, PGKK (PGKM for pRePX plasmid). It involved the substitution of lysine 646 with an aspartic acid (PGKM to PGDM) (Figure 3.1). Both single and double mutant vectors showed markedly reduced cytotoxicity in BHK-21 cells relative to the wild-type vector.

The observed attenuation probably results from the ability of these modified replicons to permit the synthesis of host cell proteins, a requirement for survival of infected cells. The inefficiency of inhibition of endogenous host cell proteins has been well documented in SIN and SFV virus harboring similar mutations in NsP2 (49, 101). The double mutant, V(PD), was the least cytotoxic of all the modified vectors. This was not surprising because, SIN and SFV incorporating similar mutations in the nuclear localization signal motif of NsP2, rendered NsP2 entirely cytoplasmic, resulting in significant reduction in inhibition of host cell protein synthesis and pathogenicity in mice (101, 134). The specific role of NsP2 in the nucleus remains unclear. In addition to reduced cytotoxicity, the single mutant replicons exhibited relative persistence and high-

level transgene expression within cells. The presence of such a phenotype requires ongoing minus-strand synthesis in addition to reduced cytopathogenicity. Interestingly, in the alphavirus replication cycle, minus-strand synthesis is shut-off a few hours postinfection. It is possible therefore that the mutation in the NsP2 may have deregulated the switch from minus strand to plus strand synthesis. In fact, in a SIN variant (S1) incorporating a similar mutation in NsP2, accumulation of P123 and P23 (involved in minus-strand synthesis) was reported (123). Another possible explanation is that the mutation(s) in NsP2 may lead to generation of polyprotein intermediates and mature NsP monomers with redundant functions. This proposed explanation stems from earlier studies on alphavirus replication cycle (138, 156).

The double mutant, V(PD), exhibited a delayed, low-level expression phenotype although it was the most persistent of all the mutants. It is likely that the mutations may have distorted the conformation of the replicase thereby interfering with efficient RNA processing, replication and synthesis: low levels of RNA replication have been associated with several alphavirus replicon variants with similar NsP2 mutations (54, 101, 123, 124). Since high-level replication is associated with increased double-stranded RNA intermediates, and these intermediates activate the apoptotic agent, protein kinase PKR, it is understandable that certain mutations that endow a non-cytotoxic phenotype will result in decreased replication (153).

Another interesting feature of the modified VEE replicons was that of a temperature-dependent packaging phenotype. The wild-type replicons were packaged efficiently at 37˚C, while the single mutants were packaged more efficiently at 35˚C, and the double mutant was packaged at 28˚C but not at the higher temperature. Inefficient and

temperature-sensitive packaging in NsP2-mutant vectors have been previously reported (101, 123), and may be related to improper sequestration of the RNA genomes or interference with the packaging machinery. It is worth noting that, V(PD) behaved similarly to one variant of the SFV vector, SFV(PD713P) (101), with respect to temperature-dependent packaging and transgene expression. V(PD) however, was different from another variant, SFV(PD), which showed increased packaging efficiency at 37˚C and high-level transgene expression at 31˚C. Finally, the wide range of phenotypes seen in NsP2-mutant studies, including this study, suggests the possibility of other yet unknown functions of NsP2. Studies toward uncovering new roles of NsP2 will increase our general understanding of alphavirus replication, synthesis, and assembly. Furthermore, insights into the different roles played by the various alphavirus genes may aid in the design of more efficient vaccine vectors as well as broaden the spectrum of their applications.

In conclusion, we have developed attenuated, prolonged-expression VEE replicons that could serve as improved vaccine vectors in immunogenicity studies where long-term antigen presentation is needed.

CHAPTER 4: COMPARATIVE ANALYSIS OF NEUTRALIZING ANTIBODY INDUCTION IN A MURINE AND RABBIT MODEL FOLLOWING IMMUNIZATION REGIMENS INCORPORATING WILD-TYPE AND PROLONGED-EXPRESSION VEE REPLICONS ENCODING HIV-1 ENVELOPE GLYCOPROTEIN

INTRODUCTION

Ongoing efforts to improve immunogenicity of HIV-1 vaccines have involved structural modification of viral immunogens as well as the development of new vaccine delivery strategies. Various forms of viral antigens are usually delivered as DNA vaccines, subunit vaccines, or through the use of viral vectors. Studies conducted previously in this laboratory with respect to HIV-1 vaccine development have employed the use of the VEE vector with encouraging results. These studies have established that immunization of small animals with Env from a particular strain of HIV-1 designated R2, can induce antibodies that neutralize multiple strains of HIV-1 of diverse subtypes (48, 129, 130, 178). In these previous studies, immunizations were conducted using various forms of the R2 Env. One of the forms shown to be highly immunogenic was the soluble oligomeric R2gp140. This soluble oligomeric form retains many of the functions of the the mature envelope spikes on the surface of the virus (48). In comparative immunogenicity studies performed in mice, R2gp140 induced potent cross-reactive neutralizing antibody responses compared to the R2gp120 form (Dong and Quinnan, unpublished data). To date, neutralizing antibodies induced against HIV-1 have been less potent than those required for protection. Effective protection against a broad array of primary strains of HIV-1 necessitates higher, more potent neutralizing antibodies that cross-react with many strains of virus. The limited potency of the responses achieved with the VEE replicon particles (VRPs) were unusual compared to the responses that have been reported with VRPs expressing glycoproteins of other viruses. The limited potency of the response may relate to the nature of the HIV-1 Env presented to immune cells. However, the limited potency of the responses could also be related to the dynamics

of VRP expression of HIV-1 Env *in vivo*. In immunogenicity studies using a murine model, optimal antibodies were achieved only when replicon doses where administered in clusters at short intervals (i.e., every 3 days x 3 doses) (48). This immunization approach was used to increase the duration of protein expression *in vivo*, since cells transduced by VRPs generally die within 24-48 hours due to cytopathic effects. The transient expression of the protein that is characteristic of VRP expression may not be sufficient to result in sufficiently differentiated, high affinity antibody responses.

We have recently developed variant forms of the VEE vector that are less cytotoxic and possess a prolonged-expression phenotype. This was achieved by introducing targeted mutations in the non-structural protein gene 2 (NsP2) region of the VEE genome (see Chapter 3). The creation of the variant vectors led us to design experiments to evaluate the relative effectiveness for neutralizing antibody induction of immunization regimens incorporating wild-type and/or variant forms of VEE vectors.

In this report, we describe results of immunization studies employing wild-type and modified VRPs with attenuated, prolonged-expression phenotypes, in a mouse and rabbit model, in an attempt to optimize immunogenicity toward HIV-1 Env.

RESULTS

I. Immunogenicity of wild-type and modified VRPs encoding R2gp160

Immunogenicity studies were initially conducted in C3H/He mice. In experiment I, the mice were administered gp160-expressing VRPs at weeks 0, 5, and 18. Mouse groups received either wild-type VRPs, V-R2gp160, or modified VRPs incorporating an

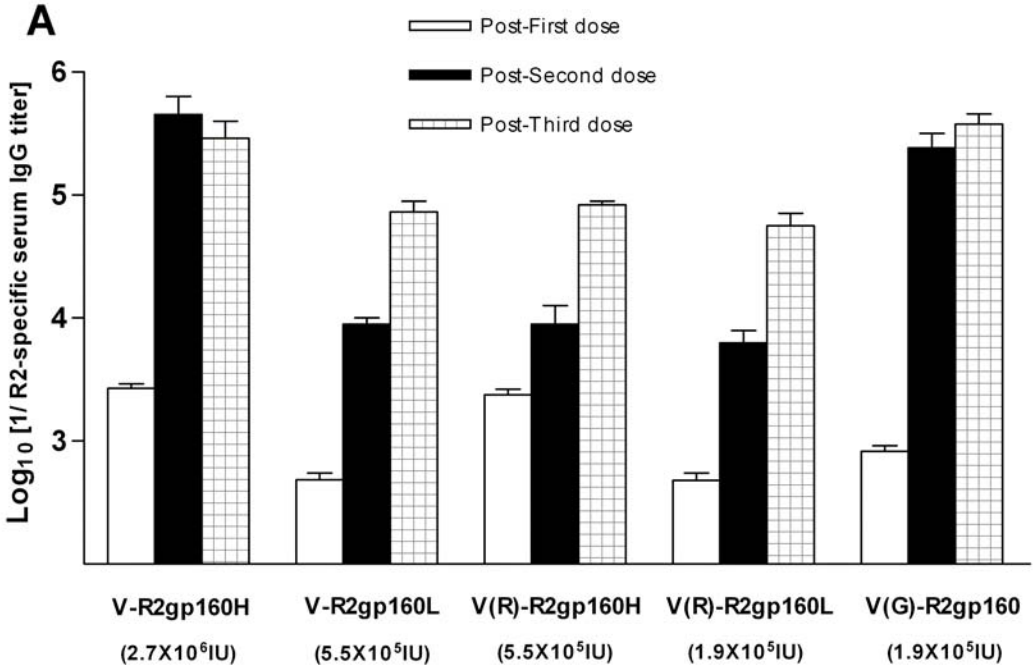
arginine or glycine mutation at proline 713, V(R)-R2gp160 and V(G)-R2gp160 respectively. The administered dose range varied due to limitations in achieving high titers of mutant VRPs similar to wild-type levels (refer to Table 4.1). The highest administered dosage of wild-type VRPs for one mouse group (V-R2gp160H) was based on a level that had induced potent immune responses in an earlier study (48). Other mouse groups received doses of either wild-type or mutant VRPs that allowed for comparison between groups (see Table 4.1). Non-immunized control mice as well as V-HeVG (VRPs expressing HeV G glycoprotein) immunized mice were maintained in parallel.

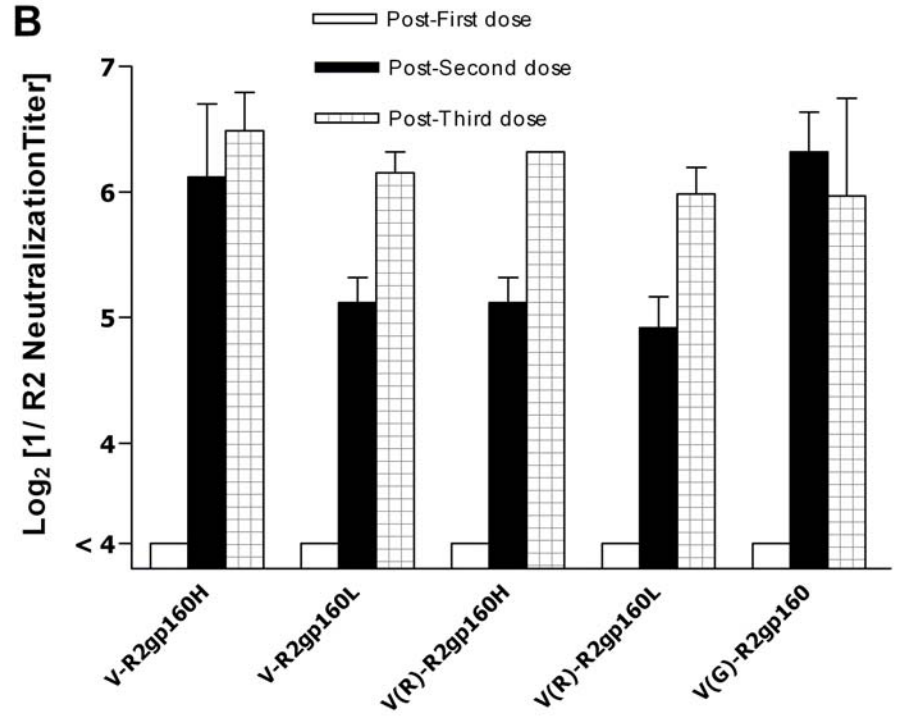
Sera from the non-immunized and V-HeVG-immunized mice did not exhibit any R2gp160-specific IgG following each of three vaccinations. These control sera were used as baseline for determining titers of R2gp160 immune sera. After second dose, substantial increases in envelope-specific antibody titers were detected in all mouse groups that received gp160-expressing VRPs. The highest response was recorded in the group that received the highest dose of VRPs, V-R2gp160H. Interestingly, the second highest response was detected in the mouse group that received the mutant VRP, V(G)-R2-gp160, even though the administered dosage in this group was three or fifteen fold lower compared to groups that received wild-type VRPs. The antibody response detected in the V(G)-R2gp160 group after the second immunization was significantly higher than the response seen in the V-R2gp160L (wild-type) group which received a three fold higher dose ($P < 0.001$) (Figure. 4.1A). No significant increases in R2-specific IgG responses were observed after administration of the third dose in both V(G)-R2gp160 and V-R2gp160H groups.

*Table 4.1: Design of experiment I: Immunization of mice with wild-type and mutant VRPs expressing HIV-1 R2gp60. * The letter in ( ) represents the resulting amino acid residue from the mutation at proline 713 in the NsP2 region of the VEE genome. ** Infectious units. *** The H and L designations represent the high and low dose respectively for a given VRP type.*

Immunogen	Transgene	Dose (IU)**	Designation***
Wild Type: V	gp160	2.7×10^6	V-R2gp160H
Wild Type: V	gp160	5.5×10^5	V-R2gp160L
Mutant: V(R)*	gp160	5.5×10^5	V(R)-R2gp160H
Mutant: V(R)*	gp160	1.9×10^5	V(R)-R2gp160L
Mutant: V(G)*	gp160	1.9×10^5	V(G)-R2gp160
Wild Type: V	V-HeVG	1.2×10^6	V-HeVG
None	N/A	N/A	Control

Figure 4.1: Comparative ELISA binding (A), and neutralizing activities (B), of sera of C3H/He mice immunized with various doses of wild-type or mutant VRPs encoding R2gp160. Doses are indicated on the horizontal axis below each group label (A). H and L represent high and low dose respectively for a given VRP type. Immunizations were given at weeks 0, 5, and 18 of study. Blood was obtained for assay 2 weeks after each immunization. (A) Binding ELISA antibodies were detected using R2gp120 protein-coated plates. (B) Neutralizing activity was measured against viruses pseudotyped with primary HIV-1 R2 Env. Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence > 50% compared to non-immunized and V-HeVG-immunized control sera at the same dilution. Titers are presented as geometric means ± SD on a log base 10 scale. $P < 0.001$ and $P < 0.05$ indicate statistically significant increases in ELISA binding and neutralizing antibodies respectively in the V(G)-R2gp160 group compared to the V-R2gp160L (wild-type) group following second immunization.


A
Post-First dose
Post-Second dose
Post-Third dose
Log10 [1/ R2-specific serum IgG titer]
6
5
4
3
V-R2gp160H
(2.7X10^6IU)
V-R2gp160L
(5.5X10^5IU)
V(R)-R2gp160H
(5.5X10^5IU)
V(R)-R2gp160L
(1.9X10^5IU)
V(G)-R2gp160
(1.9X10^5IU)


B
Post-First dose
Post-Second dose
Post-Third dose
Log2 [1/ R2 NeutralizationTiter]
7
6
5
4
< 4
V-R2gp160H
V-R2gp160L
V(R)-R2gp160H
V(R)-R2gp160L
V(G)-R2gp160

Comparable increases in antibody response were observed in the V-R2gp160L, V(R)-R2gp160H and V(R)-R2gp160L groups after the third immunization.

Neutralizing titers determined in the sera of these mice were defined as the highest dilution that resulted in ≥50% inhibition of luciferase activity. Inhibition was determined by comparison to the average result obtained for R2 pseudotyped virus treated with pooled serum from non-immunized and V-HeVG-immunized mice maintained and bled in parallel with the immunized mice. Calculations in determining the 50% neutralization titers took into account the well documented age dependent non-specific inhibitory activity seen in non-immune mouse serum. These calculations have been validated in previous studies (48, 129). For an immune serum to be considered neutralizing, the mean luminescence obtained at a particular dilution of immune serum could be no more than 50% of the mean luminescence obtained at the same dilution of non-immune and V-HeVG immune sera tested in parallel.

Neutralizing titers against the HIV-1 R2 strain were detected in all groups immunized with R2gp160-expressing VRPs after two immunizations. The V(G)-R2gp160 group exhibited neutralizing titers that were significantly higher than that seen in the V-R2gp160L (wild-type) group following the second immunization ($P < 0.05$) (Figure. 4.1B). After the third vaccination, neutralizing antibody titers were comparable among all groups.

The high potency of the immune response observed in the V(G)-R2gp160-immunized mice coupled with subsequent successes in producing high-titered mutant VRPs at lower temperatures, warranted the design of new experiments (experiment II). This allowed for head-to-head comparison of the R2gp160-expressing wild-type and

mutant VRPs at equal dosage. In addition to the wild-type and V(G)-R2gp160 VRPs used in experiment I, V(PD)-R2gp160 VRP was also included in the immunization studies carried out in experiment II. V(PD)-R2gp160 incorporates two mutations in NsP2 that allowed for increased persistence and prolonged-expression of R2gp160, albeit at low levels compared to the single mutant VRPs. Another goal of experiment II was to characterize the immune response elicited by coadministration of wild-type with each of the various mutant VRP preparations. We hypothesize that the combined approach will combine optimized levels of initial transgene expression, the immune stimulatory effects of apoptosis induced by wild-type replicons, and persistence of transgene expression in a manner that may result in enhancement of response obtained with wild-type replicon alone.

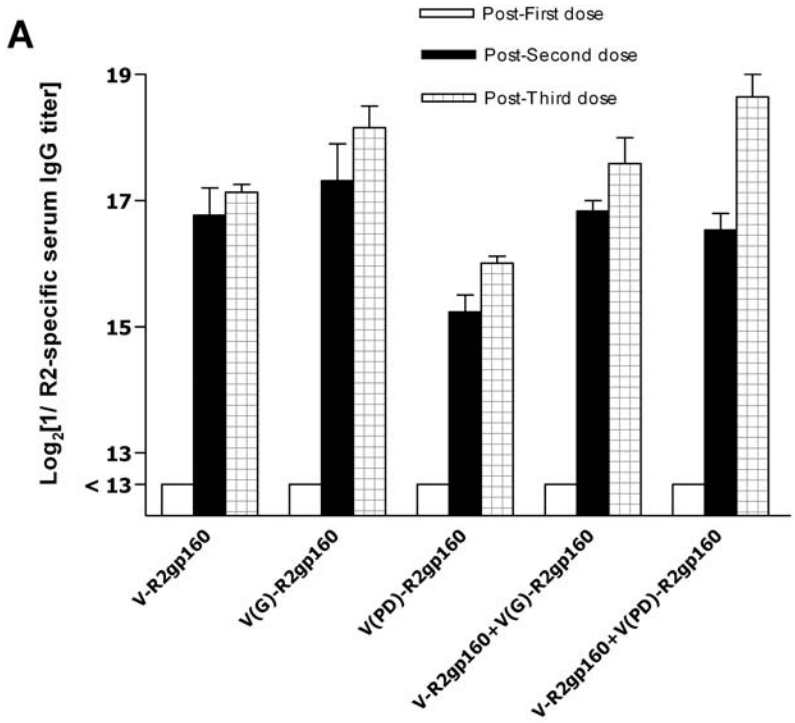
Mouse groups received either wild-type or mutant VRPs alone or a combination of wild-type and one of two mutant VRPs (see Table 4.2). The dose of V(PD)-R2gp160 VRPs administered were lower due to limitations in producing high titer VRP preparations. A total of three immunizations were administered for all groups. Non-immunized and V-HeVG-immunized control mice were maintained in parallel.

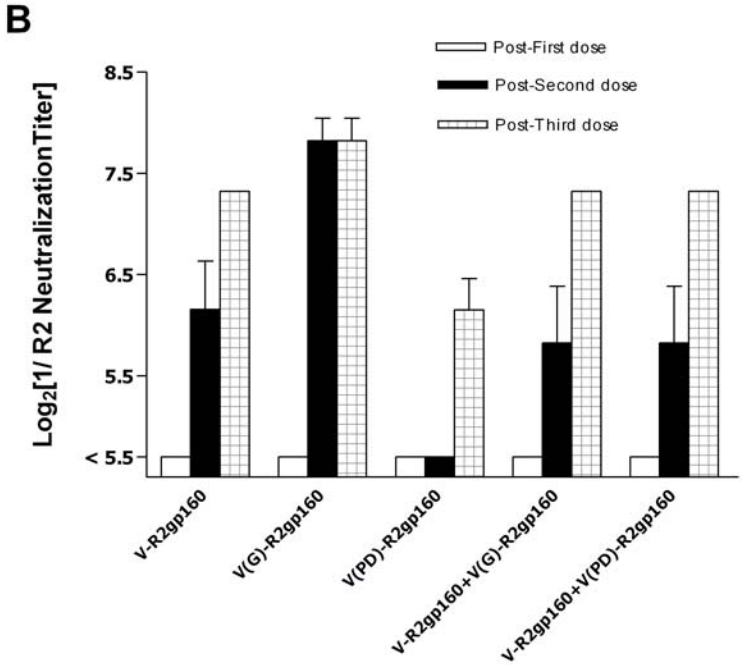
No R2gp160-specific antibodies were detected in control mice after three immunizations. After second dose, all R2gp160-immunized mice exhibited significant increases in envelope specific antibodies (Figure 4.2A). The highest antibody titers were seen in the V(G)-R2gp160 and V-R2gp160+V(PD)-R2gp160 groups following three immunizations (Figure 4.2A).

*Table 4.2: Design of Experiment II: Immunization of mice with wild-type and mutant VRPs expressing HIV-1 R2gp160. * The letter in ( ) represents the resulting amino acid residue from the mutation at proline 713 in the NsP2 region of the VEE genome. The double letters in ( ) indicate the resulting amino acid residues from mutations at alanine 259 and lysine 646 respectively in the NsP2 region. ** Infectious units.*

Immunogen	Transgene	Dose (IU)**	Designation
Wild Type: V	gp160	1×10^6	V-R2gp160
Mutant: V(G)*	gp160	1×10^6	V(G)-R2gp160
Mutant: V(PD)*	gp160	5×10^4	V(PD)-R2gp160
Wild type: V + Mutant: V(G)*	gp160	$5 \times 10^5 + 5 \times 10^5$	V-R2gp160+V(G)-R2gp160
Wild type: V + Mutant: V(PD)*	gp160	$1 \times 10^6 + 5 \times 10^4$	V-R2gp160+V(PD)-R2gp160
Wild Type: V	V-HeVG (control)*	1.2×10^6	V-HeVG
None	N/A	N/A	Control

Figure 4.2: Comparative ELISA binding (A), and neutralizing activities (B), of sera of C3H/He mice immunized with wild-type or mutant VRPs or both. Immunizations were given at weeks 0, 4, and 12 of the study. Blood was obtained for assay 2 weeks after each immunization. ELISA binding antibodies were detected using R2gp120 protein-coated plates, and neutralizing activity was measured against viruses pseudotyped with primary HIV-1 R2 Env. Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence results of > 50% compared to non-immunized and V-HeVG immunized control sera at the same dilution. Titers are presented as geometric means ± SD on a log base 2 scale. Note: Following second immunization, statistically significant differences in neutralizing titers were detected in the V(G)-R2gp160 group compared to groups that received wild-type VRPs alone or in combination ($P < 0.05$), or the group that received V(PD)-R2gp160 alone ($P < 0.001$).


A
Post-First dose
Post-Second dose
Post-Third dose
Log2[1/ R2-specific serum IgG titer]
19
17
15
13
< 13
V-R2gp160
V(G)-R2gp160
V(PD)-R2gp160
V-R2gp160+V(G)-R2gp160
V-R2gp160+V(PD)-R2gp160

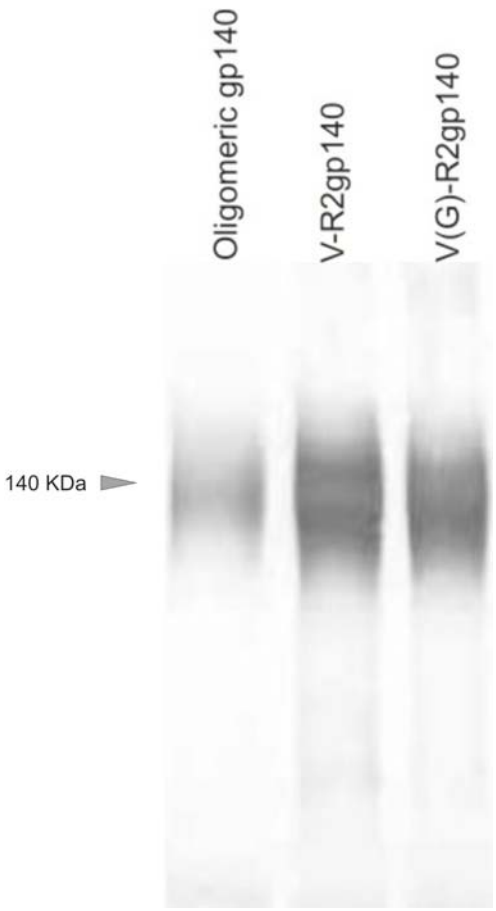

B
Post-First dose
Post-Second dose
Post-Third dose
Log2[1/ R2 NeutralizationTiter]
8.5
7.5
6.5
5.5
< 5.5
V-R2gp160
V(G)-R2gp160
V(PD)-R2gp160
V-R2gp160+V(G)-R2gp160
V-R2gp160+V(PD)-R2gp160

Sera from all R2gp160-immunized groups manifested varying degrees of neutralizing activity following three vaccinations. Importantly, following the first two vaccinations, sera from mice immunized with wild-type VRPs alone or in combination, and sera from mice immunized with V(PD)-R2gp160 alone showed significantly lower neutralizing activity than those of mice immunized with V(G)-R2gp160 alone ($P < 0.05$ and $P < 0.001$ respectively) (Figure 4.2B).

II. Immunogenicity of wild-type and modified VRPs encoding R2gp140

In experiment III, rabbit immunogenicity studies were performed to compare the wild-type VRP encoding soluble R2gp140 (V-R2gp140) with the single mutant, V(G)-R2gp140, previously shown in experiments I and II to induce high potency neutralizing antibodies. The use of the rabbit model in this experiment stems from a recent study conducted in this laboratory, evaluating an immunization regimen in rabbits (121). This regimen consisted of the soluble, oligomeric form of the Env of HIV-1 R2, R2gp140, or the surface component of the same Env, R2gp120, in the adjuvant AS02A. This study revealed that rabbits immunized with R2gp140 developed antibodies that neutralized a broad array of primary strains of HIV-1 of diverse subtypes, including some strains that are typical neutralization resistant strains (121). More so, rabbits can produce antibodies with long complementarity determining region 3 of the heavy chain (CDR H3), a feature seen in human monoclonal antibodies with broadly cross-neutralizing activity (111, 113). To confirm expression of R2gp140 by both V-R2gp140 and V(G)-R2gp140 VRPs, medium from cells infected with the respective VRPs were analyzed by western blot.

Figure 4.3: Expression of soluble HIV-1 Env, R2gp140, by wild-type and modified VRPs. BHK-21 cells were infected with wild-type or modified VRPs encoding R2gp140. At 24 hours postinfection, culture medium of infected cells were harvested and prepared for testing. Oligomeric gp140 control sample and test samples were loaded on 4 – 12% SDS-PAGE. Following transfer to nitrocellulose membrane, the blot was probed with HIV-1 positive human serum. Blot was then incubated with alkaline phosphate-conjugated goat anti-human IgG and developed.


Oligomeric gp140
V-R2gp140
V(G)-R2gp140
140 KDa

Soluble gp140 was detected in each medium and the protein migrated at the same molecular weight to the oligomeric gp140 positive control loaded with the samples (Figure 4.3).

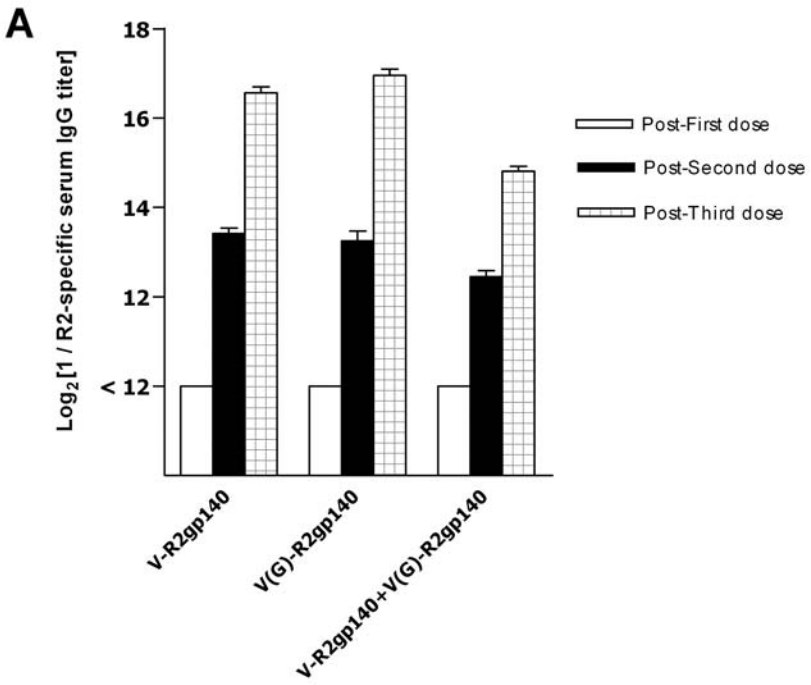
Groups of three rabbits each were immunized with either wild-type or mutant VRPs or a combination of both at 10^7 infectious units (IU) per dose (Table 4.3). Rabbits received three vaccinations similar to the immunization regimen in experiment II. Immunizations were given at weeks 0, 4 and 12. Non-immunized control rabbits were maintained throughout the study.

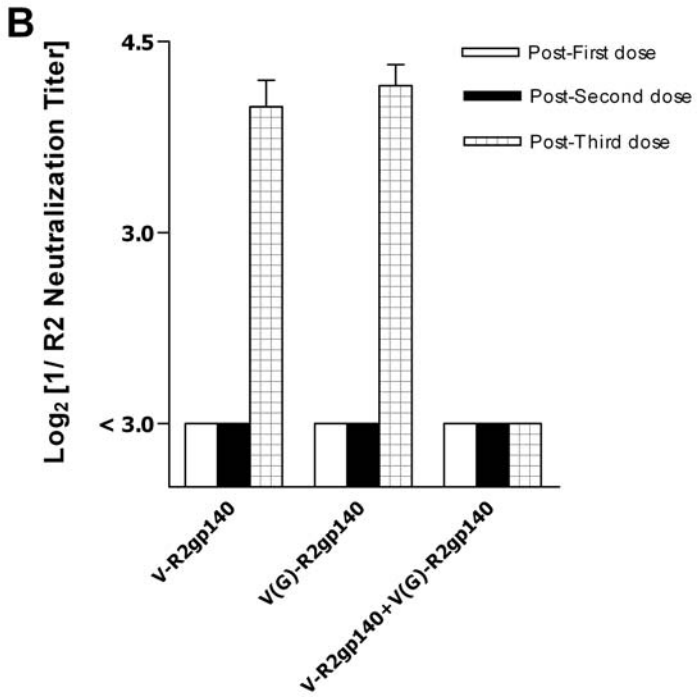
The binding antibody levels and neutralizing titers in rabbits after administration of three doses of VRPs encoding R2gp140 were lower than those seen in high potency VRP mouse immunizations (Figure. 4.4A and B). No detectable neutralizing activity developed in rabbits immunized with the wild-type and mutant combinations (Figure 4.4B) based on a starting serum dilution of 1:5.

*Table 4.3: Design of experiment III: immunization of mice with wild-type and mutant VRPs expressing HIV-1 R2gp140. * The letter in ( ) represents the resulting amino acid residue from the mutation at proline 713 in the NsP2 region of the VEE genome. ** Infectious units.*

Group	Transgene	Dose (IU)**	Designation
Wild Type: V	gp140	1×10^7	V-R2gp140
Mutant: V(G)*	gp140	1×10^7	V-R2gp140
Wild type: V + Mutant: V(G)*	gp140	$5 \times 10^6 + 5 \times 10^6$	V-R2gp140+V(G)-R2gp140
Control	N/A	N/A	Control

Figure 4.4: Immune response in rabbits immunized with wild-type and / or mutant VEE replicon constructs. Graphs illustrate comparative ELISA binding (A), and neutralization titers (B), of sera of New Zealand white rabbits immunized with wild-type or mutant VRPs or both at 10^7 infectious units per dose. Immunizations were administered at weeks 0, 4, and 12 of the study. Blood was obtained from ear veins for assay 2 weeks after each immunization. Binding antibodies were captured by R2gp140 antigen-coated plates and detected using biotinylated anti-rabbit IgG and horseradish peroxidase streptavidin. Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence results of > 50% compared to non-immunized control sera at the same dilution. Titers are presented as geometric means ± SD on a log base 2 scale.


A
18
16
14
12
< 12
Log2 [1 / R2-specific serum IgG titer]
Post-First dose
Post-Second dose
Post-Third dose
V-R2gp140
V(G)-R2gp140
V-R2gp140+V(G)-R2gp140


B
4.5
3.0
< 3.0
Log2 [1/ R2 Neutralization Titer]
Post-First dose
Post-Second dose
Post-Third dose
V-R2gp140
V(G)-R2gp140
V-R2gp140+V(G)-R2gp140

DISCUSSION

The VEE-derived vector system has emerged as an important vaccine platform and has been used extensively for induction of immune responses against emerging and re-emerging viruses (31, 34, 44, 45, 48, 63, 87, 128, 129). One of such viruses for which the VEE vector system has been utilized to induce potent immune responses has been HIV-1. Although some successes have been achieved in generating strong immune responses to HIV-1, these responses have been less than those required for protection. In a quest to augment the immune responses to HIV-1, we focused on improving the VEE vector delivery system.

We demonstrate in this report that, by using attenuated, prolonged-expression VRPs in immunization regimens, it is possible to optimize immunogenicity to HIV-1 Env. The generation of attenuated, prolonged expression vectors was accomplished by introducing single or double mutations into the NsP2 region of VEE vector genome. These mutations were based on similar mutations in alternative alphaviruses that have been shown to induce persistent, non-cytotoxic phenotypes (2, 49, 54, 101, 123, 124). The premise for using modified VRPs was based on the hypothesis that, prolonged antigen presentation to immune cells, enabled by the attenuated state of the vector, would induce sufficiently differentiated, high affinity antibody responses.

R2gp160- and R2gp140-specific responses were achieved following administration of either wild-type or mutant VRPs in both mouse and rabbit models. In experiment I, immune response to wild-type VRPs was dose-dependent, consistent with dose-response effects observed in previous studies (48, 76, 127, 128) (Figure 4.1). In contrast, sera from mice immunized with V(G)-gp160 at much lower doses exhibited

comparable or higher neutralizing activity to that seen in mice immunized with wild-type VRPs (Figure 4.1B). This result indicates high immunogenicity of attenuated, prolonged-expression VRPs. Subsequent immunizations in mice (experiment II) using equivalent doses of V-R2gp160 and V(G)-R2gp160 confirmed the superiority of the mutant VRP in the context of neutralizing antibody induction. Importantly, only two doses of mutant VRPs, V(G)-R2gp160, were needed to elicit maximum response compared to three doses for wild-type VRPs (Figure 4.2). This important attribute of mutant VRPs directly addresses an important property of all 'good' vaccines; the ability to induce the maximum effect with the least amount of inoculum by the least number of inoculations.

The low level response produced by the double mutant VRPs may not unexpected because previous studies (refer to Chapter 3) had shown prolonged, low level expression by the double mutant. It is likely that this low level expression is not optimal for induction of a strong immune response. Failure of the combined wild-type and mutant VRP inoculum to induce enhanced immune responses went contrary to our initial hypothesis. It is likely that the combined approach hampered prolonged-expression of the R2gp160 immunogen by the mutant VRPs due to rapid cytopathic effects triggered by the presence of the wild-type VRPs in the co-inoculum, thus leading to minimal or no enhancement in immunogenicity seen with mutant VRP alone

A recent report by Thompson et al., has demonstrated adjuvant effects of VRPs (159). The molecular basis for this adjuvant effect was shown to reside in the ability of VRPs to replicate and was independent of the transgene expressed (159). The mutant VRPs encoding a prolonged-expression phenotype have the ability to replicate for more sustained periods than wild-type VRPs thus making them even better adjuvants. Although

the double mutant, V(PD)-R2gp160 did not induce optimal responses, it was previously shown to be the most persistent within host cells. It is worth evaluating the possibility of boosting the overall immune response to the HIV-1 Env by immunizing small animals with wild-type, single and double mutant VRPs at separate sites. The expectation is that, this comprehensive approach will combine the immune stimulatory effects of apoptosis induced by wild-type VRPs, the optimized levels of transgene expression by single mutant VRPs, and the sustained replication by double mutant VRPs in a manner that may result in enhancement of response obtained with wild-type or single mutant VRPs alone.

The low level neutralizing activity seen in the rabbit study is consistent with the kinetics of antibody development seen in responses induced by gp140 in an earlier study conducted in this laboratory (121). In that study, antibody development induced by gp140 in rabbits tended to be more gradual compared to the rapid gp120-induced responses. The gp140-induced responses tended to increase after the fourth dose of immunogen, and were associated with more broadly cross-reactive neutralizing activity against diverse HIV-1 strains compared to gp120-induced responses. Based on this earlier report, it is likely that additional immunization of rabbits in this study may lead to similar cross-reactive, broad spectrum neutralizing activity. Neutralizing activity of sera of rabbits in this study was tested only against homologous virus. It is likely that other HIV-1 strains may be sensitive to neutralization by sera from the study rabbits.

In summary, we have developed an HIV-1 vaccine based on a modified VEE vector platform, and have demonstrated that the delivery of HIV-1 immunogens using this variant form of the vector is more effective than with wild-type vector. Attenuated,

prolonged-expression variants of VEE and other alternate alphavirus vectors should be considered when developing alphavirus-based vaccines.

CHAPTER 5: INDUCTION OF NEUTRALIZING ANTIBODIES TO HENDRA AND NIPAH GLYCOPROTEINS USING A MODIFIED VENEZUELAN EQUINE ENCEPHALITIS *IN VIVO* EXPESSION SYSTEM

INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) are important human pathogens that have emerged only recently. HeV and NiV are the prototype members of a new genus, *Henipavirus*, in the *Paramyxoviridae* family, and are also zoonotic BSL-4 priority pathogens. NiV was first described in 1998 during an outbreak in Malaysia and was primarily transmitted to humans from infected pigs. The outbreak was responsible for 256 cases of encephalitis, with a nearly 40% mortality rate (35, 36, 92). Subsequent NiV outbreaks have been documented in Bangladesh from 2001 to 2005 and were associated with increased incidence of acute respiratory distress syndrome in conjunction with encephalitis (5-7, 80). Higher case fatality rates, approaching 75%, and person-to-person transmission were observed with the more recent NiV outbreaks (5-7, 80). HeV emerged in Australia in 1994 and was identified as the cause of fatal respiratory disease in horses, which in turn was transmitted to humans causing a fatal pulmonary disease (52, 168). Both HeV and NiV are of particular concern for biodefense. NiV has been classified as a category C select agent and unlike other viral biodefense agents, they can be readily isolated from natural sources (37, 133, 161) and are easily grown in cell culture or embryonated eggs (40). Henipaviruses are highly infectious and pathogenic, are transmitted easily via the respiratory tract, and can infect and be transmitted to humans from livestock (52, 79). Being newly described, there is limited but growing knowledge about the biology of these viruses. There are currently no approved therapeutic treatment regimens or vaccines available for henipaviruses. To this end, substantial efforts are being made in developing protective vaccines against these viruses. Vaccine efforts to date include the use of recombinant vaccinia virus expressing the attachment (G) and

fusion (F) glycoproteins of NiV (68, 157). This approach has been shown to induce NiV-neutralizing antibodies in mouse and hamster animal models (68, 157). A canarypox virus-based vector encoding F and G glycoproteins of NiV has been shown to protect animals against NiV challenge in a pig model (167). Recently, a subunit vaccine approach utilizing purified soluble versions of G glycoproteins (sG) from HeV and NiV protected cats from NiV challenge (108). For an effective vaccine to be developed against these potential biodefense agents, vaccination would have to induce both systemic and mucosal immune responses.

An *in vivo* expression system derived from Venezuelan equine encephalitis virus (VEE) has been shown to elicit protective mucosal and systemic immunity against a variety of viral diseases (8, 70, 87, 128, 144). In this study we have employed the VEE-based vector, which packages genomic VEE replicon expressing a transgene into VRPs. These VRPs were used to induce immune responses to HeV and NiV in a murine model. Our primary objective was to determine the effectiveness of VRP for induction of antibodies that neutralize HeV and NiV. In addition to the wild-type vector, we included a modified version of the VEE replicon for VRP construction in immunogenicity experiments conducted in this study. This modified replicon is one of the variant forms previously described in an earlier Chapter (Chapter 3). The variant was generated by introduction of a single mutation in the NsP2 region of its genome. This mutation was made based on similar mutations that have been shown in alternative alphaviruses to encode a non-cytopathic phenotype (2, 49, 54, 123). The mutation involves a substitution at proline 713 in NsP2 for a glycine, and determines an attenuated prolonged-expression phenotype. In this case, prolonged expression is relative to the wild-type vector, 3-4 days

versus 1-2 days. The purpose of constructing the prolonged expression vector was to determine whether the change would result in enhanced immunogenicity. Thus, the inclusion of the modified vector in immunization regimens allowed for comparative analysis of the effectiveness of both wild-type and mutant vectors. Overall, the VEE-based vaccine approach takes advantage of the vector's inherent ability to deliver immunologic proteins to immune cells as well as their potential for induction of mucosal and systemic immunity.

RESULTS

I. Expression of HeV and NiV glycoproteins

Packaged VEE replicon particles (VRPs) were initially tested for their ability to express encoded envelope proteins of HeV and NiV. BHK-21 cells were simultaneously coinfected with V-HeVF and V-HeVG or V-HeVF and V(G)-HeVG or V-NiVF and V-NiVG combinations. Cell lysates were prepared 24 h postinfection and probed by western blot. Using cross-reactive anti-NiVF and anti-sHeVG antibodies, respectively, expression of the F and G glycoproteins of both viruses by the corresponding wild-type or mutant VRPs were confirmed. A ~ 60 kDa F_0 protein and a ~ 70-75 kDa HeVG protein were detected for both henipaviruses consistent with previously published data (Figure 5.1) (19).

Figure 5.1: Identification of F and G proteins of HeV and NiV expressed in BHK-21 cells. Cell lysates were prepared from BHK-21 cells 24 h postinfection with the different VRPs. Cell lysates were separated by 4 - 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred electrophoretically to a nitrocellulose membrane, and probed with rabbit anti-NiVF antibody (A), or anti-sHeVG antibody (B).


A
Marker
V-HeVF+V-HeVG
V-HeVF+V(G)-HeVG
V-NiVF+V-NiVG
75 KDa
50 KDa

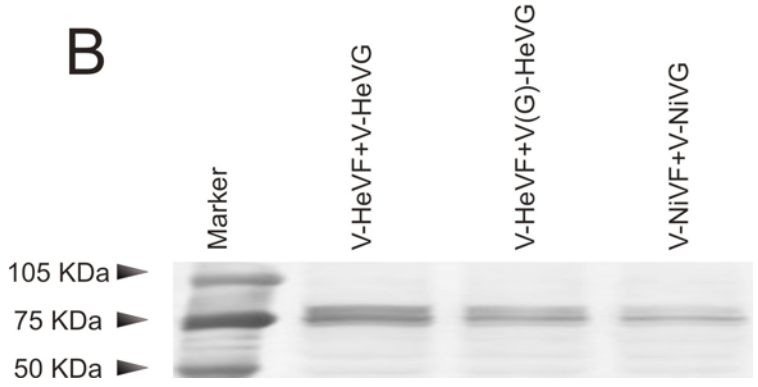
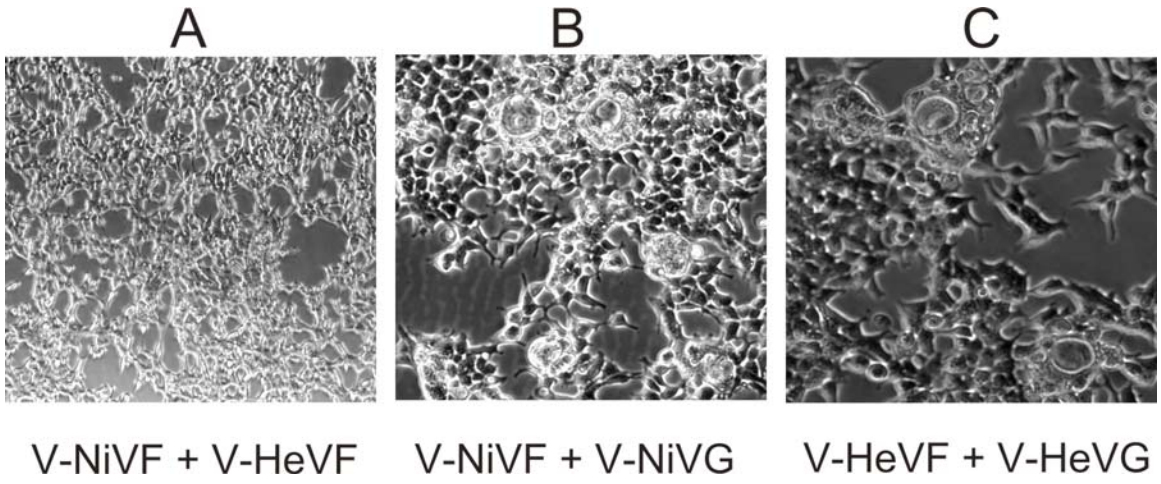

B
Marker
V-HeVF+V-HeVG
V-HeVF+V(G)-HeVG
V-NiVF+V-NiVG
105 KDa
75 KDa
50 KDa

Figure 5.2: Syncytium formation in HEK 293T cells transiently expressing F and G glycoproteins of HeV or NiV. HEK 293T cells were transfected with V-NiVF +V-HeVF (A), V-NiVF + V-NiVG (B), or V-HeVF + V-HeVG (C). Photomicrographs were taken 36 h after transfection.


A
B
C
V-NiVF + V-HeVF
V-NiVF + V-NiVG
V-HeVF + V-HeVG

An effect of live henipavirus infection of mammalian cells is syncytia formation, mediated by the coexpression of viral F and G glycoproteins. To determine whether the VRP-expressed proteins were biologically functional, V-HeVF and V-HeVG or V-NiVF and V-NiVG combinations were used to infect 293T cells. Syncytia were observed in each case with the exception of control cells which were coinfected with V-NiVF and V-HeVF (Figure 5.2).

II. Immunogenicity of G glycoprotein-expressing VRPs

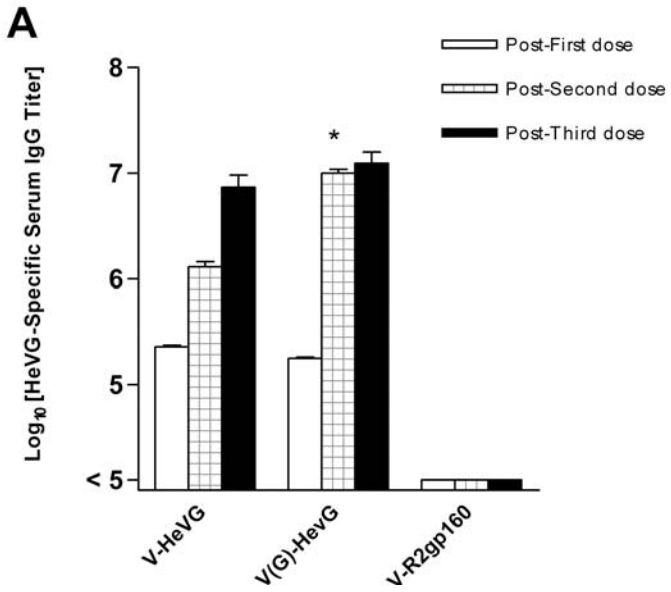
To evaluate the potential of binding and neutralizing antibody induction by G glycoprotein-expressing VRPs, immunogenicity studies were conducted in C3H/He mice. Included in the immunization regimen were wild-type VRPs expressing HeVG or NiVG (V-HeVG and V-NiVG respectively), and modified prolonged-expression VRPs expressing HeVG (V(G)-HeVG) (see Table 5.1) The inclusion of V(G)-HeVG allowed for comparison with V-HeVG in terms of the magnitude of immune response elicited. We hypothesized that V(G)-HeVG would induce stronger immune responses by increasing the duration of protein presentation to immune cells.

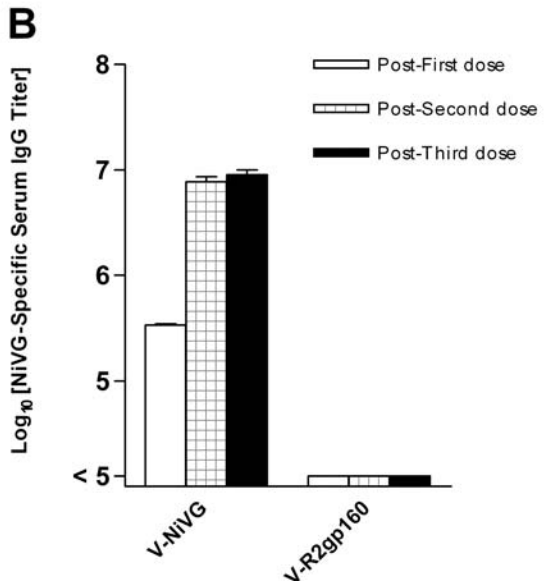
Groups of five mice were immunized by footpad inoculation with either 1.2×10^6 IU of V-HeVG or V(G)-HeVG or 3.1×10^5 IU of V-NiVG, or 2.7×10^6 IU V-R2gp160 (VRP encoding a nonrelevant HIV-1 Env.) (Table 5.1). A non-immunized control group was also maintained throughout the study. The reason for the lower inoculation dose of V-NiVG was due to the lower titers achieved in V-NiVG preparations relative to V-HeVG.

*Table 5.1: Design of Henipavirus G immunogenicity studies: Immunization of mice with wild-type VRPs expressing HeVG or NiVG. * The letter ( ) represents the resulting amino acid residue from the mutation at proline 713 in the NsP2 region of the VEE genome. ** Infectious units*

Immunogen	Transgene	Dose (IU) **	Designation
Wild Type: V	HeVG	1.2×10^6	V-HeVG
Mutant: V(G)*	HeVG	1.2×10^6	V(G)-HeVG
Wild type: V	NiVG	3.1×10^5	V-NiVG
Wild type: V	R2gp160	2.7×10^6	V-R2gp160
None	N/A	N/A	Control

*Figure 5.3: Antibody response in mice immunized by footpad inoculation with replicon constructs encoding HeVG (A) or NiVG (B). Groups of C3H/He mice (n = 6) were immunized on weeks 0, 5, and 18 with 1.2 x 10^6 IU or 3.1 x 10^5 IU of replicon particles encoding HeVG or NiVG respectively. Control mice received 2.7 x 106 IU of V-R2gp160. HeVG- and NiVG-specific antibody titers were measured by ELISA on weeks 2, 7, and 20. Bars show GM titers ± SD on a log base 10 scale. * Titers were significantly higher after two doses in the mouse group that received V(G)-HeVG ($P < 0.05$) compared to the V-HeVG group.*


A
Post-First dose
Post-Second dose
Post-Third dose
Log10 [HeVG-Specific Serum IgG Titer]
8
7
6
5
< 5
*
V-HeVG
V(G)-HevG
V-R2gp160

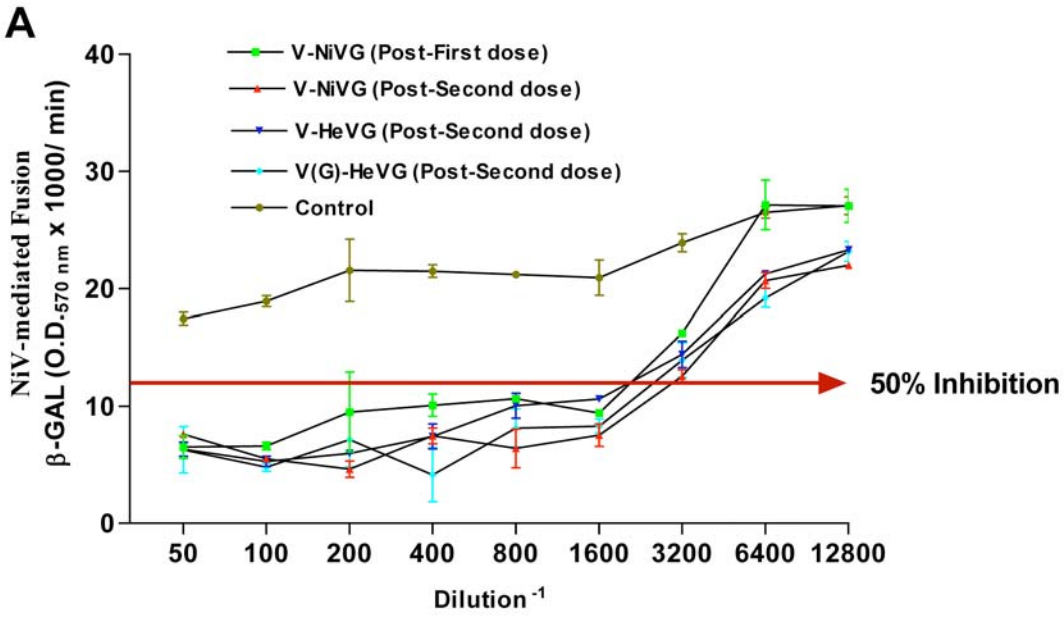

B
Post-First dose
Post-Second dose
Post-Third dose
Log10 [NiVG-Specific Serum IgG Titer]
8
7
6
5
< 5
V-NiVG
V-R2gp160

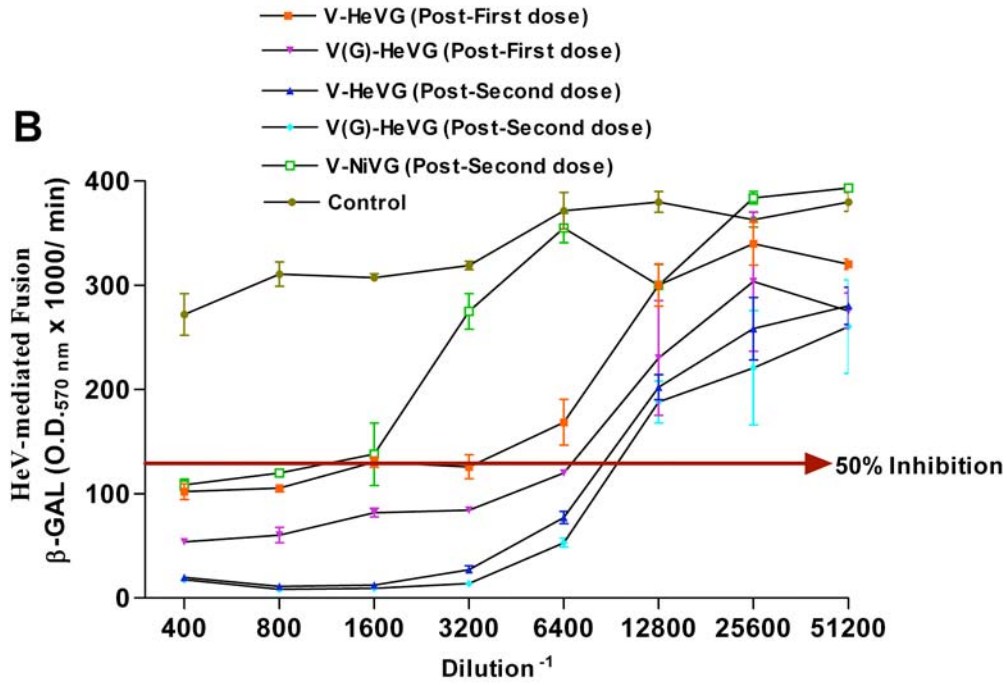
It is possible that we may be underestimating the titers of V-NiVG since titrations of VRPs were performed using anti-sHeVG antibodies. Mice in each group received three doses, one each at weeks 0, 5 and 18. All mice were bled two weeks after each dose.

There were no detectable henipavirus G-specific antibodies in sera of mice immunized with V-R2gp160. Following the second immunization, there was a significant increase in geometric mean (GM) titers in all three groups (V-HeVG, V(G)-HeVG and V-NiVG). Titers were significantly higher after two doses in the mouse group that received the modified VRPs, V(G)-HeVG ($p < 0.05$), compared to V-HeVG group (Figure 5.3A), but titers were comparable in these two groups after the third dose. Titers in the V-NiVG group were near maximal after two doses (Figure 5.3B).

Sera from these mice were subsequently analyzed for their ability to inhibit cell-cell fusion (described in material and methods) (14, 16-19). V-NiVG immune sera post-first and -second dose inhibited NiV-mediated fusion by 50% at dilutions up to 1:3200 (Figure 5.4A). After the first and second doses, sera from V-HeVG and V(G)-HeVG groups were able to inhibit HeV mediated fusion by 50% at dilutions greater than 1:6400 (Figure 5.4B). The 50% inhibition titers for post-second dose sera from mice in the V-HeVG group were less than two fold higher compared to post-first dose sera of mice in the V(G)-HeVG group. Following second immunization V(G)-HeVG sera inhibited slightly more than V-HeVG sera although this difference was not statistically significant. Cross reactivity was observed with HeV and NiV immune sera with regards to their ability to inhibit NiV- and HeV-mediated cell-cell fusion respectively (Figure 5.4A and B).

Figure 5.4: Quantitative NiV- and HeV-mediated fusion inhibition assay. HeLa cells were infected with recombinant vaccinia viruses encoding either NiV F and G or HeV F and G, along with recombinant vaccinia viruses encoding T7 RNA polymerase (effector cells). PCI target cells were infected with the reporter vaccinia virus vCB21R, encoding E. coli lacZ. NiV (A), or HeV (B) glycoprotein-expressing cells (10^5) were mixed with PCI target cells (10^5) preincubated with various sera from VRP-immunized mice, in duplicate wells of a 96-well plate. After 3 h at 37˚C, Nonidet P-40 was added and β-Gal activity was quantified.


A
V-NiVG (Post-First dose)
V-NiVG (Post-Second dose)
V-HeVG (Post-Second dose)
V(G)-HeVG (Post-Second dose)
Control
50% Inhibition
NiV-mediated Fusion
β-GAL (O.D.570 nm x 1000/ min)
Dilution -1
0
10
20
30
40
50
100
200
400
800
1600
3200
6400
12800


B
V-HeVG (Post-First dose)
V(G)-HeVG (Post-First dose)
V-HeVG (Post-Second dose)
V(G)-HeVG (Post-Second dose)
V-NiVG (Post-Second dose)
Control
50% Inhibition
HeV-mediated Fusion
β-GAL (O.D.570 nm x 1000/ min)
Dilution -1
0
100
200
300
400
400
800
1600
3200
6400
12800
25600
51200

Next, we tested the neutralizing activity of the various sera (described in Materials and Methods). Neutralizing antibodies were assayed using viruses pseudotyped with NiVF + NiVG glycoprotein (Figure 5.5A), HeVF + NiVG glycoproteins (Figure 5.5B) and HeVF + HeVG glycoproteins (Figure 5.5C). Sera from the HeVG and NiVG-immunized groups were able to mediate 90% neutralization of Nipah (NiVF + NiVG), Hendra (HeVF + HeVG) and heterotypically (HeVF + NiVG) pseudotyped viruses at considerably high titers (Figure 5.5A-C). The 90% neutralization titers were significantly higher ($p < 0.001$) after second dose, compared to post first dose titers. The NiVG-immune sera were less potent in neutralizing heterologous virus in comparison to HeVG-immune sera which neutralized heterologous, heterotypic and homologous virus with high potency. No significant increase in neutralization titers was observed across all groups after administration of the third vaccine dose. Sera from V-R2gp160-immunized control mice did not exhibit neutralizing activity.

III. Immunogenicity of F glycoprotein-expressing VRPs

To address the significance of F-specific immune responses mouse immunization experiments were designed. Groups of five mice were immunized by footpad inoculation with either V-HeVF or V-NiVF or V-R2gp160 (VRP encoding a nonrelevant HIV-1 Env.) (Table 5.2). Mice in each group received three doses, one each at weeks 0, 4, and 28. No henipavirus F-specific antibodies were detected in sera of mice immunized with V-R2gp160. Significant increases in HeVF- and NiVF-specific IgG titers, as measured by ELISA, were found in mice immunized with V-HeVF and V-NiVF respectively, ($P < 0.001$) following second the immunization, with no further increase after the third dose (Figure5.6A and B).

Figure 5.5: Serum 90%-neutralization titers from mice immunized by footpad inoculation with various VRPs outlined in Figure 5.3. Sera were assessed for their ability to neutralize Nipah pseudovirus (A) or heterotypic pseudovirus (virus pseudotyped with HeVF and NiVG proteins) (B) or Hendra pseudovirus (C) infection of HEK 293T cells using a pseudovirion neutralization assay. 90% neutralization titers were measured using sera collected on weeks 2, 7, and 20.after 1, 2, and 3 doses of immunogen Responses were expressed as GM titers ± SD on a log base 2 scale.

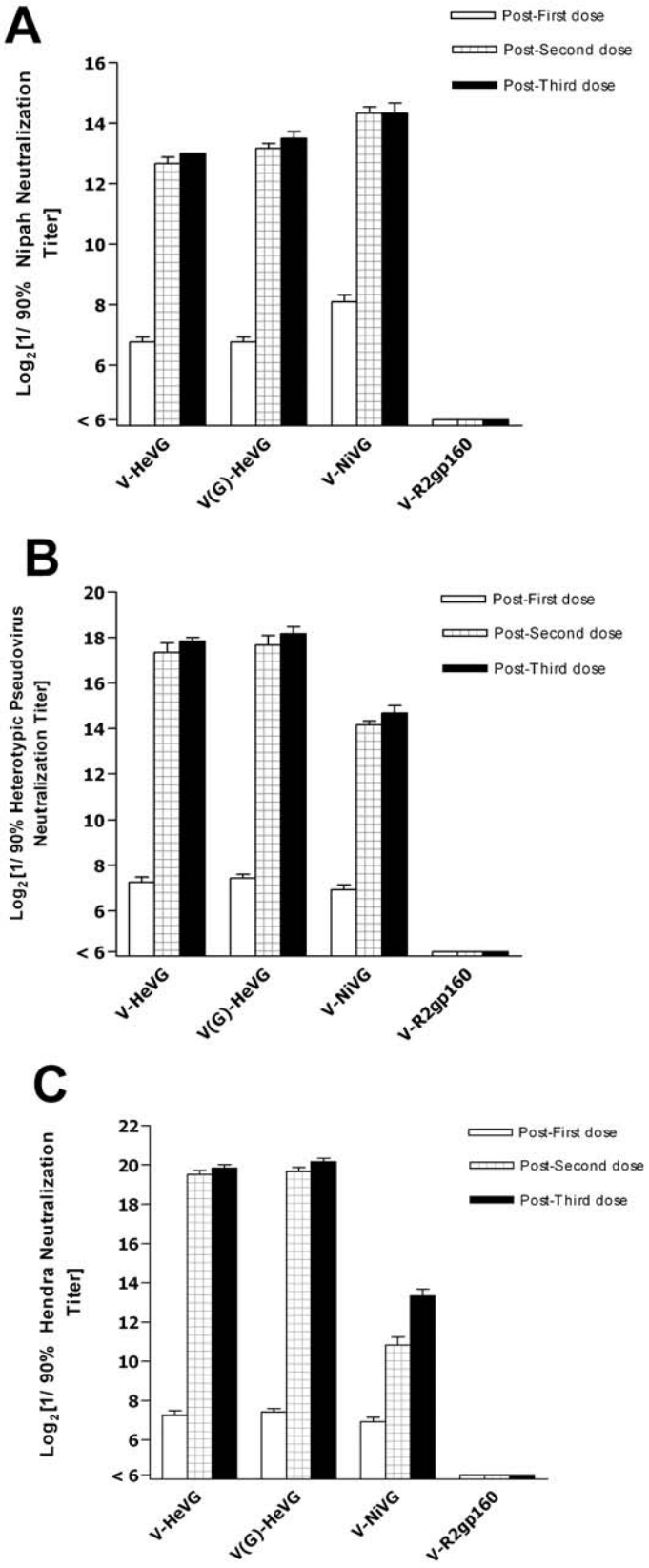
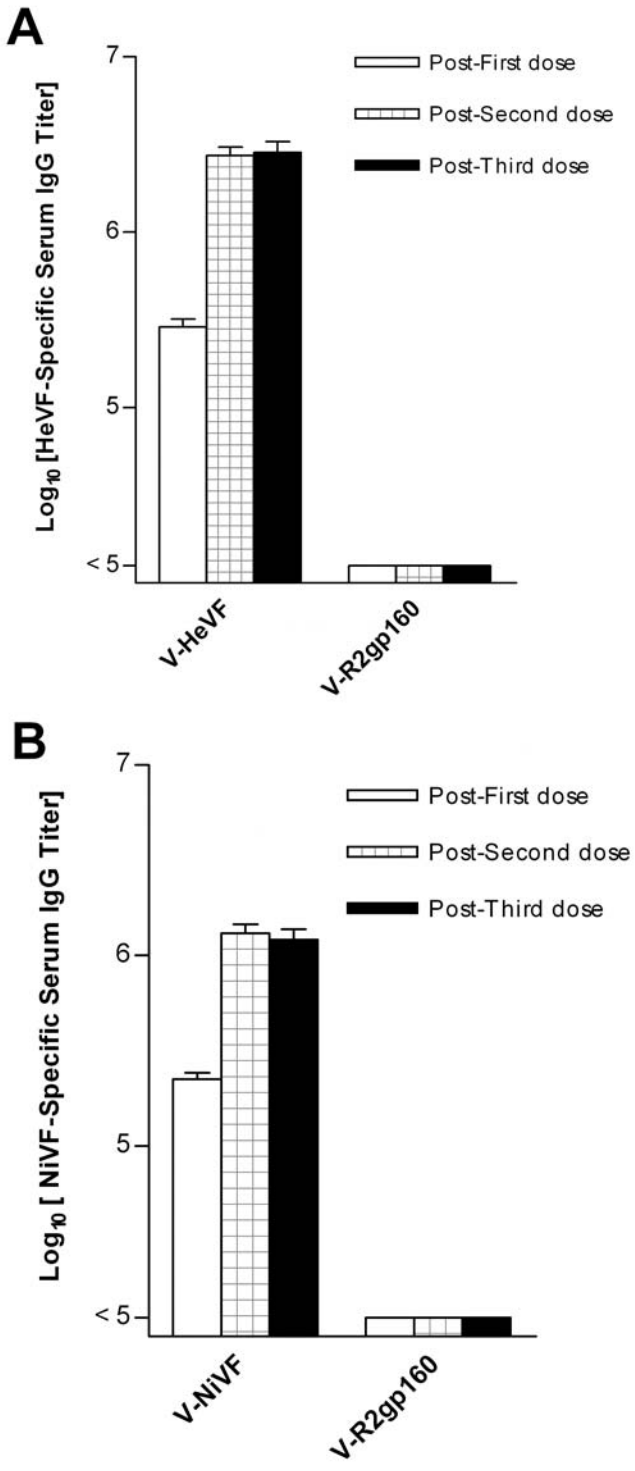

A
Post-First dose
Post-Second dose
Post-Third dose
Log2[1/ 90% Nipah Neutralization Titer]
16
14
12
10
8
6
< 6
V-HeVG
V(G)-HeVG
V-NiVG
V-R2gp160
B
Post-First dose
Post-Second dose
Post-Third dose
Log2[1/ 90% Heterotypic Pseudovirus Neutralization Titer]
20
18
16
14
12
10
8
6
< 6
V-HeVG
V(G)-HeVG
V-NiVG
V-R2gp160
C
Post-First dose
Post-Second dose
Post-Third dose
Log2[1/ 90% Hendra Neutralization Titer]
22
20
18
16
14
12
10
8
6
< 6
V-HeVG
V(G)-HeVG
V-NiVG
V-R2gp160

*Table 5.2: Design of Henipavirus F immunogenicity studies: Immunization of mice with wild-type VRPs expressing HeVF or NiVF. * Infectious units. ** VRP dose for each individual mouse per group was the same, ensured by using a fixed homogenous pool of VRP preparation for all immunizations; replicon particle titers could not be calculated due to lack of a suitable antibody for use in immunofluorescent assays.*

Immunogen	Transgene	Dose (IU) *	Designation
Wild Type: V	HeVF	50 λ VRPs **	V-HeVF
Wild Type: V	NiVF	50 λ VRPs **	V-NiVF
Wild type: V	R2gp160	1×10^6	V-R2gp160
None	N/A	N/A	Control

Figure 5.6: Antibody response in mice immunized by footpad inoculation with replicon constructs encoding HeVF (A), or NiVF (B). Groups of C3H/He mice (n = 6) were immunized on weeks 0, 4, and 28. Control mice received VRPs encoding HIV-1 R2gp160 Env. HeVF- and NiVF-specific serum IgG titers were measured by ELISA on weeks 2, 6, and 30. Bars show GM titers ± SD on a log base 10 scale.


A
Log10 [HeVF-Specific Serum IgG Titer]
7
6
5
< 5
Post-First dose
Post-Second dose
Post-Third dose
V-HeVF
V-R2gp160
B
Log10 [NiVF-Specific Serum IgG Titer]
7
6
5
< 5
Post-First dose
Post-Second dose
Post-Third dose
V-NiVF
V-R2gp160

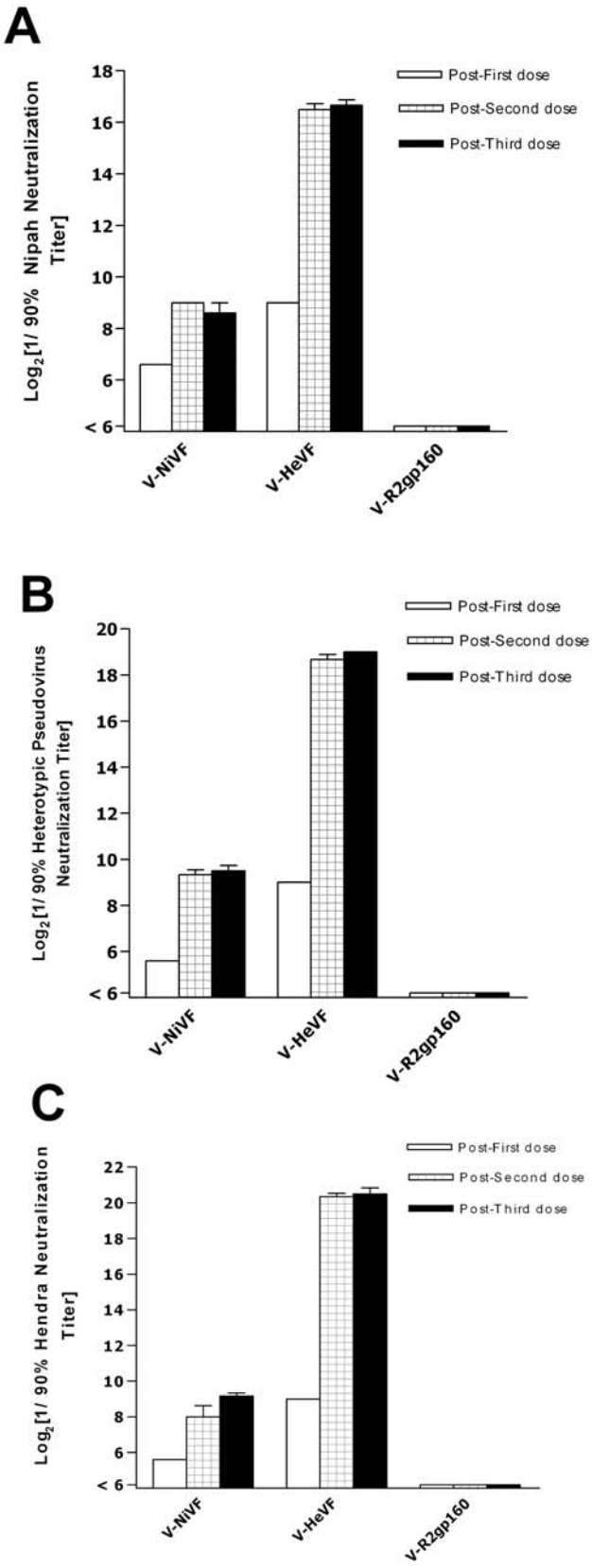
With regards to 90% neutralization titers against NiVF + NiVG (Figure 5.7A), HeVF + NiVG (Figure 5.7B) and HeVF + HeVG (Figure 5.7C) pseudotyped viruses, sera of mice in both V-HeVF and V-NiVF groups exhibited considerable neutralizing activity. Significant increases in serum neutralization titers were detected in both groups following the second immunization, and the HeVF-immune sera had the most potent neutralizing activity. No significant increases were observed after administration of the third dose. No detectable neutralizing titers were seen in the V-R2gp160 and non-immunized control groups.

DISCUSSION

The VEE replicon vector has been used with credible success to induce protective immune responses to important viral pathogens (30, 31, 43, 44, 128). We demonstrate in this report that the scope of VEE viral vectors can be extended to cover their utility in inducing cross-reactive, potent neutralizing antibodies to two zoonotic BSL-4 pathogens; HeV and NiV.

Neutralizing antibodies produced against enveloped viruses including *Paramyxoviruses* target the major envelope glycoproteins (66, 115). It is for this reason that vaccine approaches to induce protective immune responses to henipaviruses utilize the F and G envelope glycoproteins as immunogens. Henipavirus vaccine approaches that have been reported to date have utilized recombinant vaccinia or canarypox vectors, or soluble G glycoproteins to induce protective immune responses to NiV, and to some degree HeV (17, 68, 108, 167, 180). To our knowledge, this is the first time an alphavirus-derived vector system has been employed to induce immune responses to HeV and NiV.

Figure 5.7: Serum 90%-neutralization titers from mice immunized by footpad inoculation with various VRPs encoding NiVF or HeVF glycoprotein. Sera were assessed for their ability to neutralize Nipah pseudovirus (A), or heterotypic pseudovirus (virus pseudotyped with HeVF and NiVG proteins) (B), or Hendra pseudovirus (C) infection of HEK 293T cells using a pseudovirion neutralization assay. 90% neutralization titers were measured using sera collected on weeks 2, 6, and 30. Responses were expressed as GM titers ± SD on a log base 2 scale.


A
Log2[1/ 90% Nipah Neutralization Titer]
18
16
14
12
10
8
6
< 6
Post-First dose
Post-Second dose
Post-Third dose
V-NiVF
V-HeVF
V-R2gp160
B
Log2[1/ 90% Heterotypic Pseudovirus Neutralization Titer]
20
18
16
14
12
10
8
6
< 6
Post-First dose
Post-Second dose
Post-Third dose
V-NiVF
V-HeVF
V-R2gp160
C
Log2[1/ 90% Hendra Neutralization Titer]
22
20
18
16
14
12
10
8
6
< 6
Post-First dose
Post-Second dose
Post-Third dose
V-NiVF
V-HeVF
V-R2gp160

The advantages of using a VEE replicon vector platform include their targeting of dendritic cells for high level protein expression, minimal preexisting immunity to VEE in the general population, and well documented record of induction of protective immune responses in different animal models to various viral agents, as referenced above.

Optimal induction of neutralizing antibody responses against HeV and NiV will probably require presentation of biologically functional proteins to immune cells. We confirmed the functional nature of expressed F and G proteins by demonstrating induction of syncyticium formation in 293T cells coinfected with VRPs encoding F and G of either HeV or NiV. This effect documents the ability of the two expressed proteins to interact functionally with each other as well as with target cell membranes to produce membrane fusion. These are important functions of the native proteins on the surface of infectious virus particles. To assay for neutralizing antibodies in mouse sera, we employed both a fusion-inhibition assay and a Henipavirus F and G pseudotyped virus neutralization assay. The latter assay has been validated in experiments using known monoclonal antibodies and inhibitory peptides that have been shown to protect against henipavirus infections (Khetawat and Broder, unpublished). We showed in our mouse immunization experiments that all VRPs encoding the corresponding antigen were able to induce F- or G-specific binding antibody as well as neutralizing antibody responses in mice. In fact, only two doses of VRPs were needed to induce maximum neutralizing effects in all mouse groups. Serum dilutions as high as 1:1,024,000 of HeVF-immune sera were able to mediate 90% neutralization of hendra and heterotyically (HeVF + NiVG) pseudotyped viruses (Figure 5.7B). The inherent immunostimulatory properties of the VEE vector and de novo synthesis of immunogens *in vivo* may be responsible for the

magnitude of immune responses seen. The observed cross reactivity seen in sera of HeV- or NiV-immunized mice against heterologous and heterotypic virus is consistent with reports from previous studies (108, 167).

In this study, we compared the relative efficiency of induction of immune responses by VRPs incorporating wild-type and prolonged-expression mutant replicons. Although significantly higher binding antibody titers were detected in the mutant (V(G)-HeVG) group after second immunization compared to the V-HeVG group, the neutralizing activity of sera of mice in both groups was comparable. It is likely that maximal neutralizing titers were reached following administration of two doses of either V-HeVG or V(G)-HeVG. Based on the results of this study, it is likely that the F or G glycoprotein of either virus is sufficient to induce potent, cross-reactive neutralizing antibodies that may protect against henipaviruses. It remains to be seen whether immune responses produced against henipaviruses by VRPs are long lasting. Based on previous vaccine studies with alphavirus-based vectors, the immune response seen in this study should be equally long-lived.

In conclusion, we have developed HeV and NiV immunogens using the VEE replicon expression system and have demonstrated that strong immune responses to HeV and NiV glycoproteins can be achieved using this vaccine platform.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Prevention and control measures have been vital for the management of emerging and re-emerging viral diseases. Historically, vaccines have been the most efficient and cost effective means for the control of infectious diseases, resulting in the eradication of smallpox and the control of yellow fever, measles and polio. The emergence of HIV/AIDS two decades ago has brought about a surge in the variety of vaccine approaches being pursued to combat the devastating effects of this pandemic. Some of the approaches have involved recombinant subunit vaccines, peptide-based vaccines, and viral-based vectors.

With recombinant subunit vaccines, initial efforts to elicit immune responses to HIV-1 focused on the Env. Recombinant protein immunogens have been derived from both gp160 and gp120. A distinct advantage of subunit vaccines is their relative safety; however, there are limitations to their use. The antigenicity of subunit proteins is affected both by their conformation and the specific cell type used for production. Given the high degree of glycosylation of the HIV-1 Env, indeed abnormal patterns of glycosylation of the recombinant envelope made in unsuitable cell lines induce low neutralizing antibodies (88).

Peptide-based vaccine approaches offer the advantage of targeting specific epitopes that lie within conserved area of the virus. Without a complete knowledge of the correlates of protective immunity these vaccines run the risk of eliciting narrowly targeted immune responses. Further, peptides administered without adjuvant are unlikely to be immunogenic (21)

Viral vectors address most of these limitations of subunit and peptide-based vaccines. Viral recombinant vaccines mimic the types of antigen presentation that occurs

during natural viral infection, and thereby offer advantages that include the potential to present antigens in their native conformation. Replication of these viral vectors *in vivo* may itself carry an adjuvant effect, which increases the value of these vectors for use in vaccine development. Although some viral vectors have seen limited use due to presence of preexisting immunity in the general population (e.g. adenoviral vectors), the alphavirus-based vector has been used extensively in animal and early human studies with credible success (30, 31, 44, 48, 76, 127, 128). Immunization studies conducted previously in this laboratory utilized VEE vaccine vectors for induction of neutralizing antibodies against HIV-1. Although these results were encouraging, it revealed areas for improvement in both the viral immunogen and delivery vector.

The focus of this thesis was to address certain limitations of the VEE-based vector, by evaluating the relative effectiveness of wild-type and improved VEE-based vectors in inducing neutralizing antibody responses, and to employ the VEE-based vector for induction of antibodies that neutralize HeV and NiV.

In the first part of our studies, previous studies in SIN and SFV were instructive and guided our approach in generating, attenuated prolonged-expression VEE vectors. Although non-cytopathic and temperature-sensitive mutations have been described in alphaviruses previously, this thesis, for the first time, provides phenotypic descriptions of effects of mutations made in proline 713, alanine 259, and lysine 646 of VEE NsP2 replicon (Chapter 3). Each of these mutations showed some similarities and at times, differences with similar mutations in SIN and SFV (see Chapter 3). Similarities between mutant VEE vectors in this study and mutant SIN and SFV vectors with similar mutations included reduced cytotoxicity and prolonged transgene expression. However, the double

mutant in our study, V(PD) was different from the mutant, SFV(PD) which possessed similar mutations. The V(PD) exhibited a delayed, expression phenotype at 37˚C and was packaged more efficiently at 28˚C. In contrast, the SFV(PD) expressed at high levels at 37˚C and was efficiently packaged at both temperatures. Interestingly, an SFV mutant with a triple mutation, SFV(PD713P) possessed similar expression and packaging characteristics to the V(PD) mutant.

The concept of generating persistent, long-term expression vectors has been around for sometime, and has seen utility in protein kinetic studies, gene therapy, signal transduction studies, and more recently antisense and RNA interference studies. However, the generation of attenuated, prolonged-expression alphavirus-based vectors for the purpose of optimizing and enhancing immune responses in immunogenicity studies is a new approach. We demonstrate in this thesis that modified VRPs with the NsP2 (P713G) mutation are efficient in optimizing neutralizing antibody responses to viral glycoproteins (Chapter 4). Only two doses were needed to induce maximal neutralizing antibody responses to HIV-1 glycoprotein compared to three doses for wild-type VRPs (Chapter 4).

Finally, this thesis provides evidence to support the fact that potent, cross-reactive neutralizing antibodies can be induced against henipaviruses using a VEE-based vector. To our knowledge, this is the first time an alphavirus-derived vector system has been used to induce immune responses to HeV and NiV.

Results generated throughout the course of this thesis project in combination with recent published reports in the field of alphavirology have led to new questions that would have to be addressed.

First, future research geared towards elucidating the role of the NsP genes in replication, synthesis and assembly would provide vital information for developing more prolonged-expression vectors. The NsP2 protein encodes the protease activity of the replicase and regulates viral replication and synthesis. It has also been directly implicated in cellular transcriptional shutoff (58, 59, 61). Therefore, systematic mutation studies with initial focus on the NsP2 region would serve as a good starting point.

Second, development of chimeric alphavirus-based vectors with sequences derived from other alphaviruses that are known to be non-pathogenic in humans is a worthwhile endeavor. The chimeric VRPs packaged with VEE envelope glycoproteins might mitigate safety concerns related to using VEE sequences, while maintaining immunogenic components provided by the lymphotropic VEE envelope proteins.

Third, results from experiments undertaken in Chapter 3 showed that co-administration of wild-type and modified replicons at the same site did not have any advantage over wild-type VRPs alone. This may suggest that the wild-type VRPs rapidly induce cytopathic effects at the site of inoculation leading to only a brief period of transgene expression. Future experiments can be designed whereby immunization with wild-type and mutant replicons can be administered at different sites. Inoculation of wild-type and mutant VRPs in separate sites would ensure that rapid cytopathic effects triggered by the wild-type VRPs would not interfere with prolonged expression of immunogen by mutant VRPs. This design would combine the benefits of high initial transgene expression and immune stimulation by apoptosis-inducing wild-type VRPs and the prolonged-expression and replicative adjuvant effects of mutant VRPs.

Finally, preliminary results not reported in this thesis indicate that mice immunized with V-HeVF and V-HeVG developed lower neutralizing titers compared to mice immunized with V-HeVF or V-HeVG alone. This result could be an artifact created by the lack of proper controls, or a consequence of co-administration of both VRPs at the same site. The latter may lead to syncytia in the area of inoculation (due to co-expression of the F and G glycoproteins) thus limiting the amount of protein expressed. It is also possible that immunization with HeVF and HeVG may have a suppressive effect on the generation of neutralizing antibodies. Such an effect has previously been seen with measles virus, another paramyxovirus. In those studies, immunization with the fusion and hemagglutinin proteins together did not result in an additive effect with respect to neutralizing titers (126, 151). Similar studies have not been reported for HeV. Thus, future studies can build on preliminary data generated in the course of this project. In such studies, mouse groups would be immunized with VRPs encoding HeVF or HeVG alone, or HeVF+HeVG combination, ensuring equal doses of each immunogen and administration at separate sites for each.

In conclusion, alphavirus-derived vectors provide promise for use in vaccines against many current and newly emerging infectious viral diseases including HIV-1, henipaviruses and other agents of biodefense concern, to which there are no approved vaccines. Future research to optimize and adapt this vaccine platform to various viral models would be valuable.

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